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(54) Title: TECHNETIUM-99m LABELED PEPTIDES FOR THROMBUS IMAGING

(57) Abstract

This invention relates to radiolabeled reagents that are scintigraphic imaging agents for imaging sites of thrombus formation *in vivo*, and methods for producing such reagents. Specifically, the invention relates to reagents each comprised of a specific binding compound, capable of binding to at least one component of a thrombus, covalently linked to a radiolabel-binding moiety. The invention provides these reagents, methods and kits for making such reagents, and methods for using such reagents labeled with technetium-99m to image thrombus sites in a mammalian body.

# TECHNETIUM-99m LABELED PEPTIDES FOR THROMBUS IMAGING

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

This invention relates to radiodiagnostic reagents and methods for producing labeled radiodiagnostic agents. Specifically, the invention relates to reagents that can be labeled with technetium-99m (Tc-99m), methods and kits for making and radiolabeling such reagents, and methods for using such reagents to image sites of thrombus formation in a mammalian body.

### 2. Description of the Related Art

Thrombosis and thromboembolism, in particular deep vein thrombosis (DVT) and pulmonary embolism (PE), are common clinical conditions that are associated with significant morbidity and mortality. It has been estimated that in the U.S. approximately 5 million patients experience one or more episodes of DVT per year and that over 500,000 cases of PE occur, resulting in 100,000 deaths (J. Seabold, Society of Nuclear Medicine Annual Meeting 1990). It has also been estimated that over 90% of all pulmonary emboli arise from DVT in the lower extremities. Foretunately, anticoagulant therapy can effectively treat these conditions, if applied early enough. However, such treatment is associated with risks (*e.g.* internal bleeding) that preclude unnecessary prophylactic application. More advanced techniques of thrombolytic intervention (such as the administration of recombinant tissue plasminogen activator or streptokinase) can be used in acute cases, but these techniques carry even greater risks. Moreover, effective clinical application of these techniques requires that the site of the offending thrombus be identified so as to monitor the effectiveness of treatment.

For these reasons, a rapid means of localizing thrombi *in vivo*, most preferably using non-invasive methods, is highly desirable. Methods currently utilized for the identification of thrombolytic sites are contrast venography and compression B-mode ultrasound; the choice of which technique is used depends

on the expected location of the thrombus. However the former technique is invasive, and both techniques are uncomfortable for the patient. In addition, these methods are in many cases either unsuitable or yield inaccurate results.

In the field of nuclear medicine, certain pathological conditions are localized, or their extent is assessed, by detecting the distribution of small quantities of internally-administered, radioactively-labeled tracer compounds (called radiotracers or radiopharmaceuticals). Methods for detecting these radiopharmaceuticals are known generally as imaging or radioimaging methods.

In radioimaging, the radiolabel is a gamma-radiation emitting radionuclide and the radiotracer is located using a gamma-radiation detecting camera (this process is often referred to as gamma scintigraphy). The imaged site is detectable because the radiotracer is chosen either to localize at a pathological site (termed positive contrast) or, alternatively, the radiotracer is chosen specifically <u>not</u> to localize at such pathological sites (termed negative contrast).

A number of factors must be considered for optimal radioimaging in humans. To maximize the efficiency of detection, a radionuclide that emits gamma energy in the 100 to 200 keV range is preferred. To minimize the absorbed radiation dose to the patient, the physical half-life of the radionuclide should be as short as the imaging procedure will allow. To allow for examinations to be performed on any day and at any time of the day, it is advantageous to have a source of the radionuclide always available at the clinical site.

A variety of radionuclides are known to be useful for radioimaging, including $^{67}$Ga, $^{99m}$Tc (Tc-99m), $^{111}$In, $^{123}$I, $^{125}$I, $^{169}$Yb or $^{186}$Re. Tc-99m is a particularly preferred radionuclide because it emits gamma radiation at 140 keV, it has a physical half-life of 6 hours, and it is readily available on-site using a molybdenum-99/technetium-99m generator.

Radioimaging, specifically gamma scintigraphy, provides a non-invasive method for detecting the location of thrombi *in vivo*. A gamma-emitting radiotracer that binds specifically to a component of a thrombus in preference

to other tissue when administered *in vivo* can provide an external scintigraphic image which defines the location of the thrombus-bound radiotracer and hence the thrombus.

There are several potential radiotracer targets in thrombi. Thrombi are constructs of blood cells, largely platelets, enmeshed in cross-linked fibrin protein. Venous thrombi are fibrin-rich, whereas arterial thrombi are platelet-rich. Fibrin and platelets are thus obvious targets for designing radiopharmaceuticals for imaging thrombi, each having multiple possible target sites.

Activated platelets and fibrin have been used as targets in radioimaging thrombi because neither are normally found in circulating blood; circulating blood contains unactivated platelets and fibrinogen, a fibrin precursor. Thrombus formation involves the proteolytic conversion of fibrinogen to fibrin and the physiological conversion of unactivated platelets to an activated state. Since little fibrin circulates in the bloodstream (in contrast to its precursor, fibrinogen) and since most circulating platelets are unactivated, fibrin and activated platelets are excellent and specific targets for imaging thrombi because they will not be found to any substantial extent anywhere *in vivo* other than in a thrombus.

The use of radiolabeled fibrinogen and radiolabeled platelets for radioimaging has a number of disadvantages, however. Blood and background tissue clearance of radiolabeled fibrinogen and platelets are slow, which necessitates a long delay between injection and imaging. Also, as thrombi age radiolabeled platelets become less efficient imaging agents, although fibrin and platelets already in an existing thrombus remain targets even in aged thrombi.

Attempts to provide radiotracers for imaging thrombi are known in the prior art. These include autologous platelets, labeled with either $^{111}$In or $^{99m}$Tc (Tc-99m), and $^{123}$I- and $^{125}$I-labeled fibrinogen (the latter detected with a gamma scintillation probe as opposed to a gamma camera). In addition, other thrombus-associated components of the coagulation system, such as enzymes (*e.g.* thrombin), proenzymes and other factors may be useful as

thrombus-associated targets for radiotracers. Additional radiolabeled compounds used to label thrombi include plasmin, plasminogen activators, heparin, fibronectin, fibrin Fragment $E_1$ and anti-fibrin and anti-platelet monoclonal antibodies [*see* Knight, 1990, Sem. Nucl. Med. 20: 52-67 for review].

Of the methods of radiolabeling thrombi known in the prior art, the methods that have shown the most promise are radiolabeled platelets, radiolabeled antibodies and radiolabeled fibrin Fragment $E_1$. All of these have serious drawbacks with regard to their routine use.

The use of radiolabeled autologous platelets to image thrombi requires that autologous blood be drawn, the platelets then separated and radiolabeled under sterile conditions (in addition, radiolabeling must be performed so as to avoid activating the platelets), and the radiolabeled platelets then readministered to the patient. Such radiolabeled platelets have a long circulating time, resulting in poor target to non-target ratios at early times, and thereby requiring that radioimaging be performed only after a delay of 24 to 72 hours. Moreover, aged thrombi are poorly visualized since such thrombi do not efficiently incorporate fresh platelets.

Radiolabeled antifibrin and antiplatelet monoclonal antibodies have also been used in the prior art (typically to image DVT). The disadvantage to using such reagents is that antibodies (and even antibody fragments) have slow blood and general tissue clearance characteristics and require a delay of at least several hours for optimum imaging. In addition, immunological reagents have the capacity to induce an immune response in the patient. Further, such reagents must be prepared from mammalian cell lines (hybridomas) and thus carry the risk of contamination by infectious human viruses.

Methods of using radiolabeled proteins and proteolytic fragments thereof for imaging thrombi have been described in the prior art. For example, Fragment $E_1$ is a proteolytic fragment of fibrin that is derived from coagulated, cross-linked fibrin. It has been labeled with $^{123}I$ and Tc-99m to provide high quality images in humans.

Olexa *et al.*, 1982, European Patent Application No. 823017009 disclose

pharmaceutically acceptable radiolabeled prpteolytic fragments selected from Fragment $E_1$ isolated from cross-linked fibrin, Fragment $E_2$ isolated from cross-linked fibrin, and proteolytic fragments having amino acid sequences intermediate between Fragments $E_1$ and $E_2$. Unfortunately, these protein fragments must be laboriously prepared from human fibrinogen, making them unsuitable for routine manufacture.

Hadley *et al.*, 1988, PCT/US88/03318 disclose a method for detecting a fibrin-platelet clot *in vivo* comprising the steps of (a) administering to a patient a labeled attenuated thrombolytic protein, wherein the label is selectively attached to a portion of the thrombolytic protein other than the fibrin binding domain; and (b) detecting the pattern of distribution of the labeled thrombolytic protein in the patient.

Sobel, 1989, PCT/US89/02656 discloses a method to locate the position of one or more thrombi in an animal using radiolabeled, enzymatically inactive tissue plasminogen activator.

Peptides having the ability to bind to thrombi are known in the prior art.

Ruoslahti & Pierschbacher, U.S. Patent No. 4,578,079 describe peptides of sequence X-Arg-Gly-Asp-R-Y, wherein X and Y are either H or an amino acid, and R is Thr or Cys, the peptides capable of binding to thrombi *in vivo*.

Ruoslahti & Pierschbacher, U.S. Patent No. 4,792,525 describe peptides of sequence Arg-Gly-Asp-X, wherein X is Ser, Thr or Cys, the peptides capable of binding to thrombi *in vivo*.

Klein *et al.*, 1992, U.S. Patent No. 5,086,069 disclose guanine derivatives that bind to the GPIIb/IIIa receptor, found on the cell surface of activated platelets.

Pierschbacher *et al.*, 1989, PCT/US88/04403 disclose conformationally-restricted RGD-containing peptides for inhibiting cell attachment to a substratum.

Hawiger *et al.*, 1989, PCT/US89/01742 relates to peptides comprising sequences for two binding sites of a protein.

Nutt *et al.*, 1990, European Patent Application 90202015.5 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90202030.4 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90202031.2 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90202032.0 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90311148.2 disclose cyclic peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90311151.6 disclose cyclic peptides that are fibrinogen receptor antagonists.

Ali *et al.*, 1990, European Patent Application 90311537.6 disclose cyclic peptides that are fibrinogen receptor antagonists.

Barker *et al.*, 1991, PCT/US90/03788 disclose cyclic peptides for inhibiting platelet aggregation.

Pierschbacher *et al.*, 1991, PCT/US91/02356 disclose cyclic peptides that are fibrinogen receptor antagonists.

Egbertson *et al.*, 1992, European Patent Application 0478328A1 disclose tyrosine derivatives that bind with high affinity to the GPIIb/IIIa receptor.

Ojima *et al.*, 1992, 204th Meeting, Amer. Chem. Soc. Abst. 44 disclose synthetic multimeric RDGF peptides useful in inhibiting platelet aggregation.

Hartman *et al.*, 1992, J. Med. Chem. 35: 4640-4642 describe tyrosine derivatives that have a high affinity for the GPIIb/IIIa receptor.

Radiolabeled peptides useful for radioimaging thrombi have been reported in the prior art.

Ranby *et al.*, 1988, PCT/US88/02276 disclose a method for detecting fibrin deposits in an animal comprising covalently binding a radiolabeled compound to fibrin.

Stuttle, 1990, PCT/GB90/00933 discloses radioactively labeled peptides

containing from 3 to 10 amino acids comprising the sequence arginine-glycine-aspartic acid (RGD), capable of binding to an RGD binding site *in vivo*.

Rodwell *et al.*, 1991, PCT/US91/03116 disclose conjugates of "molecular recognition units" with "effector domains".

Maraganore *et al.*, 1991, PCT/US90/04642 disclose a radiolabeled thrombus inhibitor comprising (a) a inhibitor moiety; (b) a linker moiety; and (c) an "anion binding exosite (ABE)" binding site moiety.

The use of chelating agents for radiolabeling polypeptides, and methods for labeling peptides and polypeptides with Tc-99m are known in the prior art and are disclosed in co-pending U.S. Patent Applications Serial Nos. 07/653,012, 07/807,062, 07/871,282, 07/886,752, 07/893,981, 07/955,466, 08/019,864 and 08/044,825, and PCT International Applications PCT/US92/00757, PCT/US92/10716, PCT/US93/02320 and PCT/US93/_____, which are hereby incorporated by reference.

There remains a need for small (to enhance blood and background tissue clearance), synthetic (to make routine manufacture practicable and to ease regulatory acceptance) molecules radiolabeled with Tc-99m for use in imaging thrombi *in vivo*. Small synthetic peptides radiolabeled with Tc-99m that bind specifically to components of thrombi fulfill this need and are provided by this invention.

## SUMMARY OF THE INVENTION

The present invention provides radioactively-labeled reagents that are scintigraphic imaging agents. Specifically, the invention provides reagents for preparing thrombus imaging agents that are radiolabeled with technetium-99m (Tc-99m). The reagents of the invention are each comprised of a specific binding compound, including but not limited to peptides, that binds specifically to a thrombus *in vivo*, and that is covalently linked to a radiolabel-binding moiety.

In preferred embodiments, the invention provides reagents wherein the specific binding compounds are linear or cyclic peptides having an amino acid sequence of 4 to 100 amino acids.

It is of distinct commercial advantage to use small compounds, preferably having a molecular weight of less than about 10,000 daltons. Such small compounds can be readily manufactured. Moreover, they are likely not to be immunogenic and to clear rapidly from the vasculature, thus allowing for better and more rapid imaging of thrombi. In contrast, larger molecules such as antibodies of fragments thereof, or other biologically-derived peptides larger than 10,000 daltons, are costly to manufacture, and are likely to be immunogenic and clear more slowly from the bloodstream, thereby interfering with rapid diagnoses of thrombi *in vivo*.

One aspect of the invention provides a reagent for preparing a thrombus imaging agent that is capable of being radiolabeled for imaging thrombi within a mammalian body, comprising a specific binding compound that specifically binds to at least one component of a thrombus, covalently linked to a Tc-99m binding moiety of formula:

$$C(pgp)^{s}\text{-}(aa)\text{-}C(pgp)^{s}$$

wherein $C(pgp)^{s}$ is a protected cysteine and (aa) is an amino acid. In a preferred embodiment, the amino acid is glycine.

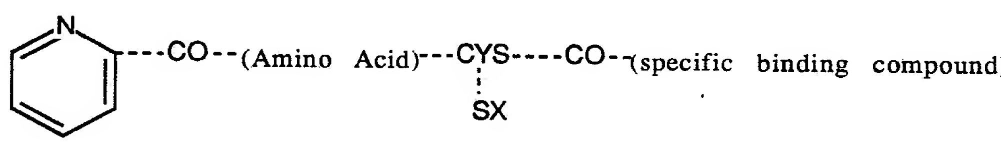
In another embodiment, the invention provides a reagent for preparing a thrombus imaging agent that is capable of being radiolabeled for imaging thrombi within a mammalian body, comprising a specific binding compound

that specifically binds to at least one component of a thrombus, covalently linked to a Tc-99m binding moiety of formula:

$$A\text{-}CZ(B)\text{-}[C(R^1R^2)]_n\text{-}X$$

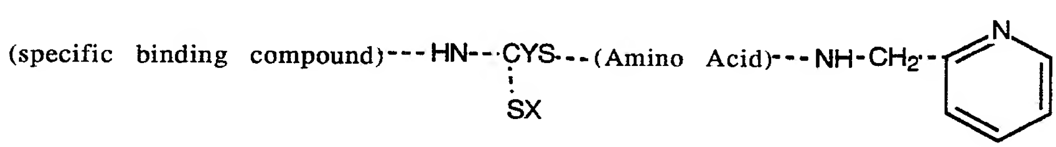
wherein A is H, HOOC, $H_2NOC$, (peptide)-NHOC, (peptide)-OOC or $R^4$; B is H, SH or $-NHR^3$, $-N(R^3)$-(peptide) or $R^4$; Z is H or $R^4$; X is SH or $-NHR^3$, $-N(R^3)$-(peptide) or $R^4$; $R^1$, $R^2$, $R^3$ and $R^4$ are independently H or straight or branched chain or cyclic lower alkyl; n is 0, 1 or 2; and: (1) where B is $-NHR^3$ or $-N(R^3)$-(peptide), X is SH and n is 1 or 2; (2) where X is $-NHR^3$ or $-N(R^3)$-(peptide), B is SH and n is 1 or 2; (3) where B is H or $R^4$, A is HOOC, $H_2NOC$, (peptide)-NHOC, (peptide)-OOC, X is SH and n is 0 or 1; (4) where A is H or $R^4$, then where B is SH, X is $-NHR^3$ or $-N(R^3)$-(peptide) and where X is SH, B is $-NHR^3$ or $-N(R^3)$-(peptide); (5) where X is H or $R^4$, A is HOOC, $H_2NOC$, (peptide)-NHOC, (peptide)-OOC and B is SH; (6) where Z is methyl, X is methyl, A is HOOC, $H_2NOC$, (peptide)-NHOC, (peptide)-OOC and B is SH and n is 0; and (7) where Z is SH and X is SH, n is not 0; and wherein the thiol moiety is in the reduced form.

In another embodiment, the invention provides a reagent for preparing a thrombus imaging agent that is capable of being radiolabeled for imaging thrombi within a mammalian body, comprising a specific binding compound that specifically binds to at least one component of a thrombus, covalently linked to a radiolabel binding moiety of formula:




[for purposes of this invention, radiolabel-binding moieties having this structure will be referred to as picolinic acid (Pic)-based moieties]

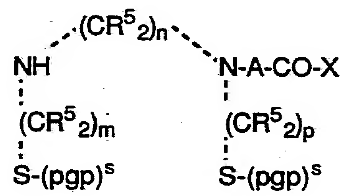
or



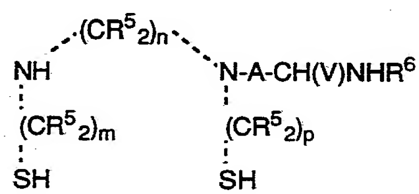

[for purposes of this invention, radiolabel-binding moieties having this structure will be referred to as picolylamine (Pica)-based moieties]; wherein X is H or a protecting group; (amino acid) is any amino acid; the radiolabel-binding moiety is covalently

linked to the peptide and the complex of the radiolabel-binding moiety and the radiolabel is electrically neutral. In a preferred embodiment, the amino acid is glycine and X is an acetamidomethyl protecting group. In additional preferred embodiments, the specific binding compound is covalently linked to the radiolabel-binding moiety *via* an amino acid, most preferably glycine.

Yet another embodiment of the invention provides a reagent for preparing a thrombus imaging agent that is capable of being radiolabeled for imaging thrombi within a mammalian body, comprising a specific binding compound that specifically binds to at least one component of a thrombusr, covalently linked to a radiolabel-binding moiety that is a bisamino bisthiol radiolabel binding moiety. The bisamino bisthiol moiety in this embodiment of the invention has a formula selected from the group consisting of:




wherein each $R^5$ can be independently H, $CH_3$ or $C_2H_5$; each $(pgp)^S$ can be independently a thiol protecting group or H; m, n and p are independently 2 or 3; A is linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof; and X is a specific binding compound, preferably a peptide; and




wherein each $R^5$ is independently H, lower alkyl having 1 to 6 carbon atoms, phenyl, or phenyl substituted with lower alkyl or lower alkoxy; m, n and p are independently 1 or 2; A is linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof; V is H or CO-peptide; $R^6$ is H or peptide; provided that when V is H, $R^6$ is peptide and when $R^6$ is H, V is peptide. [For purposes of this invention, radiolabel-binding moieties having these structures will be referred to as "BAT" moieties]. In a preferred embodiment, the specific binding compound of the reagent is covalently linked to the radiolabel-binding moiety *via* an amino acid, most preferably glycine.

In preferred embodiments of the aforementioned aspects of this invention, the specific binding compound is a peptide is comprised of between 4 and 100 amino acids. The most preferred embodiment of the radiolabel is technetium-99m.

The reagents of the invention may be formed wherein the specific binding compounds or the radiolabel-binding moieties are covalently linked to a polyvalent linking moiety. Polyvalent linking moieties of the invention are comprised of at least 2 identical linker functional groups capable of covalently bonding to specific binding compounds or radiolabel-binding moieties. Preferred linker functional groups are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups. In preferred embodiments, the polyvalent linking moieties are comprised of *bis*-succinimdylmethylether (BSME), 4-(2,2-dimethylacetyl)benzoic acid (DMAB), *tris*(succinimidylethyl)amine (TSEA), *N*-[2-(*N*_,*N*_-*bis*(2-succinimidoethyl) aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide (BAT-BS), 4-(O-$CH_2$CO-Gly-Gly-Cys.amide)acetophenone (ETAC) and *bis*-succinimidohexane (BSH).

The invention also provides thrombus imaging agents for imaging a thrombus within a mammalian body comprising a specific binding peptide having an amino acid sequence of 4 to 100 amino acids and a technetium-99m binding moiety covalently linked to the specific binding peptide, wherein the peptide is selected from the group consisting of linear and cyclic peptides that are ligands for a GPIIb/IIIa receptor and do not comprise the amino acid sequence (arginine-glycine-aspartate), peptides that are ligands for a polymerization site of fibrin, and cyclic peptides comprising the amino acid sequence (arginine-glycine-aspartate). In a preferred embodiment, the amino acid sequence of peptides that are ligands for a polymerization site of fibrin comprise multiple copies of the sequence (glycyl-prolyl-arginyl-prolyl).

The invention also comprises scintigraphic imaging agents that are complexes of the reagents of the invention with Tc-99m and methods for radiolabeling the reagents of the invention with Tc-99m. Radiolabeled complexes provided by the invention are formed by reacting the reagents of the invention with Tc-99m in the presence of a reducing agent. Preferred reducing agents include but are not limited to dithionite ion, stannous ion and ferrous ion. Complexes of the invention are also formed by labeling the reagents of the invention with Tc-99m by ligand exchange of a prereduced Tc-99m complex as provided herein.

The invention also provides kits for preparing scintigraphic imaging agents that are the reagents of the invention radiolabeled with Tc-99m. Kits for labeling the reagents of the invention with Tc-99m are comprised of a sealed vial containing a

predetermined quantity of a reagent of the invention or mixtures thereof and a sufficient amount of reducing agent to label the reagent with Tc-99m.

This invention provides methods for preparing reagents of the invention by chemical synthesis *in vitro*. In preferred embodiments, peptides are synthesized by solid phase peptide synthesis.

This invention provides methods for using scintigraphic imaging agents that are Tc-99m labeled reagents for imaging a thrombus within a mammalian body by obtaining *in vivo* gamma scintigraphic images. These methods comprise administering an effective diagnostic amount of Tc-99m labeled reagents of the invention and detecting the gamma radiation emitted by the Tc-99m label localized at the thrombus site within the mammalian body.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides reagents, including peptide reagents, for preparing radiolabeled thrombus imaging agents for imaging a thrombus within a mammalian body. The reagents provided by the invention comprise a radiolabel binding moiety covalently linked to a specific binding compound that is capable of binding to at least one component of a thrombus. For purposes of the invention, the term thrombus imaging reagent will refer to embodiments of the invention comprising a specific binding compound covalently linked to a radiolabel binding moiety and radiolabeled, preferably with Tc-99m, $^{111}$In or $^{68}$Ga, most preferably with Tc-99m.

Labeling with Tc-99m is an advantage of the present invention because the nuclear and radioactive properties of this isotope make it an ideal scintigraphic imaging agent. This isotope has a single photon energy of 140 keV and a radioactive half-life of about 6 hours, and is readily available from a $^{99}$Mo-$^{99m}$Tc generator. Another advantage of the present invention is that none of the preferred radionuclides are toxic, in contrast to other radionuclides known in the art (*for example*, $^{125}$I).

In the Tc-99m binding moieties and compounds covalently linked to such moieties that contain a thiol covalently linked to a thiol protecting groups [(pgp)$^S$] provided by the invention, the thiol-protecting groups may be the same or different and may be but are not limited to:

-$CH_2$-aryl (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-CH-(aryl)$_2$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-C-$(aryl)_3$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);
-$CH_2$-(4-methoxyphenyl);
-CH-(4-pyridyl)$(phenyl)_2$;
-$C(CH_3)_3$
-9-phenylfluorenyl;
-$CH_2NHCOR$ (R is unsubstituted or substituted alkyl or aryl);
-$CH_2$-NHCOOR (R is unsubstituted or substituted alkyl or aryl);
-CONHR (R is unsubstituted or substituted alkyl or aryl);
-$CH_2$-S-$CH_2$-phenyl

Preferred protecting groups have the formula -$CH_2$-NHCOR wherein R is a lower alkyl having 1 and 8 carbon atoms, phenyl or phenyl-substituted with lower alkyl, hydroxyl, lower alkoxy, carboxy, or lower alkoxycarbonyl. The most preferred protecting group is an acetamidomethyl group.

Each specific-binding peptide-containing embodiment of the invention is comprised of a sequence of amino acids. The term amino acid as used in this invention is intended to include all L- and D- amino acids, naturally occurring and otherwise. Reagents comprising specific-binding peptides provided by the invention include but are not limited to the following (the amino acids in the following peptides are L-amino acids except where otherwise indicated):

<u>Ligands for the GPIIb/IIIa Receptor</u>

```
OC---Y_D--RG--D
 :            :
H2C----S------C---C_Acm GC_Acm.amide

OC---Y_D--RG--D
 :            :
H2C----S------C---WGGC_Acm GC_Acm.amide

OC---Y_D--RG--D
 :            :
H2C----S------C--- FGGC_Acm GC_Acm.amide

OC---Y_D--RG--D
 :            :
H2C----S------C---GGGC_Acm GC_Acm.amide

OC---Y_D--RG--D
 :            :
H2C----S------C---DGGC_Acm GC_Acm.amide
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OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---GGG$C_{Acm}$G$C_{Acm}$.amide

OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---Apc.GDFK$C_{Acm}$G$C_{Acm}$.amide

OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---KG$C_{Acm}$G$C_{Acm}$.amide

OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---GG$C_{Acm}$G$C_{Acm}$GGC.amide

OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---GG$C_{Acm}$G$C_{Acm}$GG$C_{NES}$.amide

$\left\{ \begin{array}{l} \text{OC---}Y_D\text{--Apc.G-D} \\ H_2\text{C----S--------C---GG}C_{Acm}\text{G}C_{Acm}\text{GGC.amide} \end{array} \right\}_3$ -TSEA

OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---GG$C_{Acm}$G$C_{Acm}$GG$F_D$PRPG.amide

OC---$Y_D$--KG--D
$H_2$C----S-------C---GGG$C_{Acm}$G$C_{Acm}$.amide

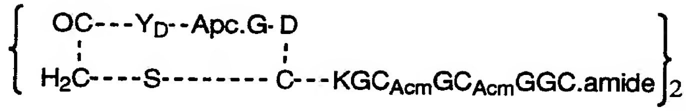
OC---$Y_D$--Apc.G-D
$H_2$C----S--------C---KG$C_{Acm}$G$C_{Acm}$GGC.amide

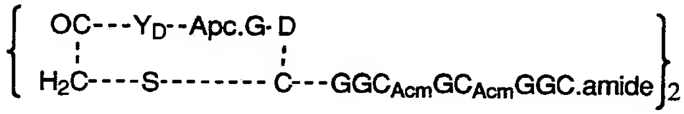
Pic.G$C_{Acm}$GG-C-NP.Apc.GDC
S————S

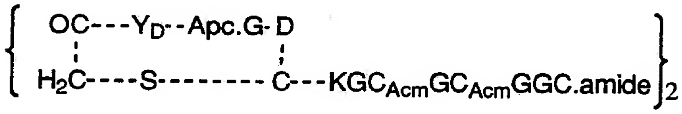
*acetyl* - $C_{Acm}$G$C_{Acm}$-C-NP.Apc.GDC
S————S

$\left\{ \begin{array}{l} \text{OC---}Y_D\text{--Apc.G-D} \\ H_2\text{C----S--------C---GG}C_{Acm}\text{G}C_{Acm}\text{GGC.amide} \end{array} \right\}_2$ -BSH

$\left\{ \begin{array}{l} \text{OC---}Y_D\text{--Apc.G-D} \\ H_2\text{C----S--------C---GG}C_{Acm}\text{G}C_{Acm}\text{GGC.amide} \end{array} \right\}_2$ -BSME


-BSME


-[BAT-BS]


-[BAT-BS]

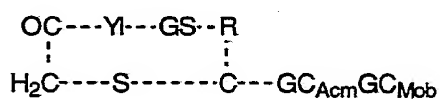
C$_{Acm}$GC$_{Acm}$GGRGDS
C$_{Acm}$GC$_{Acm}$GGRGDGGRGDS
C$_{Acm}$GC$_{Acm}$GGRGDGGRGDGGRGDS
CKRARGDDMDDYC
C$_{Acm}$GC$_{Acm}$RRRRRRRRRGDV
GRGDVKC$_{Acm}$GC$_{Acm}$.amide
GRGDVC$_{Acm}$GC$_{Acm}$.amide
GRGDVRGDFKC$_{Acm}$GC$_{Acm}$.amide
GRGDVRGDFC$_{Acm}$GC$_{Acm}$.amide
*mmp*-GGGRGDF
*acetyl*-CNP.Apc.GDC
*acetyl*-RGDC.amide
CRGDC
GRGDFGGC$_{Acm}$
*ma*$_{Bz}$-GGRGDF
C$_{Acm}$GGGRGDF
GRGDGGGGC
GRGDGGC$_{Acm}$
*ma*-GGRGDF
*ma*$_{Acm}$-GGGRGDF
*ma*-RGDF
*ma*-RGD
*acetyl*-G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide
G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide
G.Apc.GDVKC$_{Acm}$GC$_{Acm}$.amide
*acetyl*-RRARGDDLDC$_{Acm}$GC$_{Acm}$amide

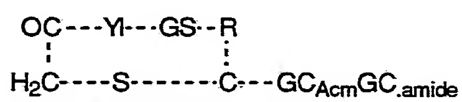
(CC$_{Acm}$GC$_{Acm}$GGRGDS)$_3$-TSEA
[Pic.SC$_{Acm}$SYNRGDSTC.amide]$_3$-TSEA
[BAT].Hly.GDP.Hly.GDF.amide
[BAT]G.Apc.GDV.Apc.GDFK.amide
CRIARGDWNDDYC
CKFFARTVCRIARGDWNDDYCTGKSSDC

<u>Thrombin Ligands</u>
C$_{Acm}$GC$_{Acm}$NDGDFEEIPEEYLQ
C$_{Acm}$GC$_{Acm}$GGF$_D$PRPGGGGNGDFEEIPEEYL
*ma*-GGGGF$_D$PRPGGGGNGDFEEIPEEYL
C$_{Acm}$GC$_{Acm}$GGF$_D$PRPGamide
(*acetyl*-F$_D$PRPG)$_2$KGGGC.amide

<u>Ligands for the Polymerization Site of Fibrin</u>
[(GPRP)$_2$K]$_2$KC$_{Acm}$GC$_{Acm}$.amide
(GPRVVERHQSA)$_2$KC$_{Acm}$GC$_{Acm}$.amide
(GPRPC$_{Acm}$GC$_{Acm}$C)$_3$-TSEA
[GPRPPPGGC$_{Acm}$GC$_{Acm}$GGC]$_3$-TSEA

<u>Derivatives of Laminin</u>





<u>Ligands for Fibrinogen</u>
CYGQQHHLGGAKQAGDV
Pic.GC$_{Acm}$GQQHHLGGAKQAGDV

<u>Derivatives of GPIIb/IIIa</u>
Pic.GC$_{Acm}$PSPSPIHPAHHKRDRRQ.amide
PSPSPIHPAHHKRDRRQC$_{Acm}$GC$_{Acm}$.amide

(Single-letter abbreviations for amino acids can be found in G. Zubay, *Biochemistry* (2d. ed.), 1988 (MacMillen Publishing: New York) p.33; other abbreviations are as in the Legend to Table I). This list of reagents provided by the

invention is illustrative and not intended to be limiting or exclusive, and it will be understood by those with skill in the art that reagents comprising combinations of the peptides diclosed herein or their equivalents may be covalently linked to any of the chelating moieties of the invention and be within its scope, including combinations of such peptides and chelating moieties comprising linking groups as disclosed herein.

In embodiments of the invention comprising peptides having an amino acid sequence that encode the platelet GPIIb/IIIa receptor, each said reagent is capable of inhibiting human platelet aggregation in platelet-rich plasma by 50% when present at a concentration of no more than 0.3μM.

Specific-binding peptides of the present invention can be chemically synthesized *in vitro*. Peptides of the present invention can generally advantageously be prepared on an amino acid synthesizer. The peptides of this invention can be synthesized wherein the radiolabel-binding moiety is covalently linked to the peptide during chemical synthesis *in vitro*, using techniques well known to those with skill in the art. Such peptides covalently-linked to the radiolabel-binding moiety during synthesis are advantageous because specific sites of covalent linkage can be determined.

Radiolabel binding moieties of the invention may be introduced into the target specific peptide during peptide synthesis. For embodiments comprising picolinic acid [(Pic-); *e.g.*, Pic-Gly-Cys(protecting group)-], the radiolabel-binding moiety can be synthesized as the last (i.e., amino-terminal) residue in the synthesis. In addition, the picolinic acid-containing radiolabel-binding moiety may be covalently linked to the E-amino group of lysine to give, for example, aN(Fmoc)-Lys-EN[Pic-Gly-Cys(protecting group)], which may be incorporated at any position in the peptide chain. This sequence is particularly advantageous as it affords an easy mode of incorporation into the target binding peptide.

Similarly, the picolylamine (Pica)-containing radiolabel-binding moiety [-Cys(protecting group)-Gly-Pica] can be prepared during peptide synthesis by including the sequence [-Cys(protecting group)-Gly-] at the carboxyl terminus of the peptide chain. Following cleavage of the peptide from the resin the carboxyl terminus of the peptide is activated and coupled to picolylamine. This synthetic route requires that reactive side-chain functionalities remain masked (protected) and do not react during the conjugation of the picolylamine.

Examples of small synthetic peptides containing the Pic-Gly-Cys- and -Cys-Gly-Pica chelators are provided in the Examples hereinbelow. This invention provides for the incorporation of these chelators into virtually any peptide capable of

specifically binding to a thrombus *in vivo*, resulting in a radiolabeled peptide having Tc-99m held as neutral complex.

This invention also provides specific-binding small synthetic peptides which incorporate bisamine bisthiol (BAT) chelators which may be labeled with Tc-99m. This invention provides for the incorporation of these chelators into virtually any peptide capable of specifically binding to a thrombus *in vivo*, resulting in a radiolabeled peptide having Tc-99m held as neutral complex. Examples of small synthetic peptide reagents containing BAT chelators as radiolabel-binding moiety is provided in the Examples hereinbelow.

In forming a complex of radioactive technetium-99m with the reagents of this invention, the technetium complex, preferably a salt of Tc-99m pertechnetate, is reacted with the reagent in the presence of a reducing agent. Preferred reducing agents are dithionite, stannous and ferrous ions; the most preferred reducing agent is stannous chloride. Means for preparing such complexes are conveniently provided in a kit form comprising a sealed vial containing a predetermined quantity of a reagent of the invention to be labeled and a sufficient amount of reducing agent to label the reagent with Tc-99m. Alternatively, the complex may be formed by reacting a reagent of this invention with a pre-formed labile complex of technetium and another compound known as a transfer ligand. This process is known as ligand exchange and is well known to those skilled in the art. The labile complex may be formed using such transfer ligands as tartrate, citrate, gluconate or mannitol, for example. Among the Tc-99m pertechnetate salts useful with the present invention are included the alkali metal salts such as the sodium salt, or ammonium salts or lower alkyl ammonium salts.

In a preferred embodiment of the invention, a kit for preparing technetium-labeled reagents is provided. An appropriate amount of the reagent is introduced into a vial containing a reducing agent, such as stannous chloride, in an amount sufficient to label the reagent with Tc-99m. An appropriate amount of a transfer ligand as described (such as tartrate, citrate, gluconate or mannitol, for example) can also be included. The kit may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. The components of the kit may be in liquid, frozen or dry form. In a preferred embodiment, kit components are provided in lyophilized form.

Radiolabeled thrombus imaging reagents according to the present invention may be prepared by the addition of an appropriate amount of Tc-99m or Tc-99m

complex into the vials and reaction under conditions described in Example 2 hereinbelow.

Radioactively-labeled scintigraphic imaging agents provided by the present invention are provided having a suitable amount of radioactivity. In forming Tc-99m radioactive complexes, it is generally preferred to form radioactive complexes in solutions containing radioactivity at concentrations of from about 0.01 millicurie (mCi) to 100 mCi per mL.

The thrombus imaging reagents provided by the present invention can be used for visualizing thrombi in a mammalian body when Tc-99m labeled. In accordance with this invention, the Tc-99m labeled reagents are administered in a single unit injectable dose. The Tc-99m labeled reagents provided by the invention may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Generally, the unit dose to be administered has a radioactivity of about 0.01 mCi to about 100 mCi, preferably 1 mCi to 20 mCi. The solution to be injected at unit dosage is from about 0.01 mL to about 10 mL. After intravenous administration, imaging of the thrombus *in vivo* can take place in a matter of a few minutes. However, imaging can take place, if desired, in hours or even longer, after the radiolabeled reagent is injected into a patient. In most instances, a sufficient amount of the administered dose will accumulate in the area to be imaged within about 0.1 of an hour to permit the taking of scintiphotos. Any conventional method of scintigraphic imaging for diagnostic purposes can be utilized in accordance with this invention.

The methods for making and labeling these compounds are more fully illustrated in the following Examples. These Examples illustrate certain aspects of the above-described method and advantageous results. These Examples are shown by way of illustration and not by way of limitation.

## EXAMPLE 1

### Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) was carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling

with dicyclohexylcarbodiimide/hydroxybenzotriazole or 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/ hydroxybenzotriazole (HBTU/HOBT), and using *p*-hydroxymethylphenoxy-methylpolystyrene (HMP) resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus amides. Resin-bound products were routinely cleaved using a solution comprised of trifluoroacetic acid, water, thioanisole (if an arginine residue comprises the peptide), ethanedithiol, and triethylsilane, prepared in ratios of 100 : 5 : 5 : 2.5 : 2 for 0.5 - 3 h at room temperature.

Where appropriate, N-terminal acetyl groups were introduced by treating the free N-terminal amino peptide bound to the resin with 20% v/v acetic anhydride in NMP (N-methylpyrrolidinone) for 30 min. Where appropriate, 2-chloroacetyl and 2-bromoacetyl groups were introduced either by using the appropriate 2-halo-acetic acid as the last residue to be coupled during SPPS or by treating the N-terminus free amino peptide bound to the resin with either the 2-halo-acetic acid/ diisopropylcarbodiimide/ N-hydroxysuccinimide in NMP of the 2-halo-acetic anhydride/ diisopropylethylamine in NMP. Where appropriate, 2-haloacetylated peptides were cyclized by stirring an 0.1 - 1.0 mg/mL solution in phosphate or bicarbonate buffer (pH 8) containing 0.5 - 1.0 mM EDTA for 4 - 48 hours, followed by acidification with acetic acid, lyophilization and HPLC purification. Where appropriate, Cys-Cys disulfide bond cyclizations were performed by treating the precursor cysteine-free thiol peptides at 0.1mg/mL in pH 7 buffer with aliquots of 0.006M $K_3Fe(CN)_6$ until a stable yellow color persisted. The excess oxidant was reduced with excess cysteine, the mixture lyophilized and then purified by HPLC.

Where appropriate the "Pic" group was introduced by using picolinic acid as the last residue in peptide synthesis. Where appropriate the "Pica" group was introduced by conjugating picolylamine to a precursor peptide using diisopropylcarbodiimide and N-hydroxysuccinimide. Where appropriate BAT ligands were introduced either by using the appropriate BAT acid as the last residue to be coupled during SPPS or by treating the N-terminus free amino peptide bound to the resin with BAT acid/ diisopropylcarbodiimide/ N-hydroxysuccinimide in NMP. Where appropriate, [BAM] was conjugated to the peptide by first activating the peptide carboxylate with a mixture of diisopropylcarbodiimide/N-hydroxysuccinimide or HBTU/HOBt in DMF, NMP or $CH_2Cl_2$, followed by coupling in the presence of diisopropylethylamine; after coupling, the conjugate was deprotected as described above.

Where appropriate, BSME adducts were prepared by reacting single thiol-

containing peptides (5 to 50 mg/mL in 50 mM sodium phosphate buffer, pH 8) with 0.5 molar equivalents of BMME (*bis*-maleimidomethylether) pre-dissolved in acetonitrile at room temperature for approximately 1-18 hours. The solution was concentrated and the product was purified by HPLC. Where appropriate, BSH adducts were prepared by using *bis*-maleimidohexane in place of BMME .

Where appropriate, TSEA adducts were prepared by reacting single thiol-containing peptide (at concentrations of 10 to 100 mg/mL peptide in DMF, or 5 to 50 mg/mL peptide in 50mM sodium phosphate (pH 8)/ acetonitrile or THF) with 0.33 molar equivalents of TMEA (*tris*(2-maleimidoethyl)amine; *see* co-pending U.S. Patent Application 07/955,466, incorporated by reference) pre-dissolved in acetonitrile or DMF, with or without 1 molar equivalent of triethanolamine, at room temperature for approximately 1-18h. Such reaction mixtures containing adducts were concentrated and the adducts were then purified using HPLC.

Where appropriate, BAT-BS adducts were prepared by reacting single thiol-containing peptide (at concentrations of 2 to 50 mg/mL peptide in 50mM sodium phosphate (pH 8)/ acetonitrile or THF) with 0.5 molar equivalents of BAT-BM (*N*-[2-(*N'*,*N'*-*bis*(2-maleimidoethyl)aminoethyl)]-$N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide; *see* co-pending U.S. Patent Application 08/044,825, incorporated by reference) pre-dissolved in acetonitrile or THF, at room temperature for approximately 1-18h. The solution was then evaporated to dryness and [BAT-BS]-peptide conjugates deprotected by treatment with 10mL TFA and 0.2mL triethylsilane for 1h. The solution was concentrated, the product adducts precipitated with ether, and then purified by HPLC.

Crude peptides were purified by preparative high pressure liquid chromatography (HPLC) using a Waters Delta Pak C18 column and gradient elution using 0.1% trifluoroacetic acid (TFA) in water modified with acetonitrile. Acetonitrile was evaporated from the eluted fractions which were then lyophilized. The identity of each product was confirmed by fast atom bombardment mass spectroscopy (FABMS).

## EXAMPLE 2

## A General Method for Radiolabeling with Tc-99m

0.1 mg of a peptide reagent prepared as in Example 2 was dissolved in 0.1 mL of water, or 50:50 ethanol:water, or phosphate-buffered saline (PBS), or 50mM potassium phosphate buffer (pH = 5, 6 or 7.4). Tc-99m gluceptate was prepared by reconstituting a Glucoscan vial (E.I. DuPont de Nemours, Inc.) with 1.0 mL of Tc-

99m sodium pertechnetate containing up to 200 mCi and allowed to stand for 15 minutes at room temperature. 25 µl of Tc-99m gluceptate was then added to the peptide and the reaction allowed to proceed at room temperature or at 100°C for 15-30 min and then filtered through a 0.2 µm filter.

The Tc-99m labeled peptide purity was determined by HPLC using the conditions described in the Footnotes in Table I. Radioactive components were detected by an in-line radiometric detector linked to an integrating recorder. Tc-99m gluceptate and Tc-99m sodium pertechnetate elute between 1 and 4 minutes under these conditions, whereas the Tc-99m labeled peptide eluted after a much greater amount of time.

The following Table illustrates successful Tc-99m labeling of peptides prepared according to Example 1 using the method described herein.

**TABLE I**

| Peptides | FABMS MH$^+$ | Radiochemical Yield(%)* | HPLC $R_T$(min)** |
|---|---|---|---|
| $CH_2CO.Y_DRGDCC_{Acm}GC_{Acm}$amide | 1057 | 97[2] | 10.0, 10.4, 10.6[2] |
| $CH_2CO.Y_DRGDCWGGC_{Acm}GC_{Acm}$amide | 1357 | 100[4] | 15.9, 16.4[2] |
| $CH_2CO.Y_DRGDCFGGC_{Acm}GC_{Acm}$amide | 1318 | 97[4] | 15.9, 16.3[2] |
| $CH_2CO.Y_DRGDCGGGC_{Acm}GC_{Acm}$amide | 1310 | 99[2] | 11.8[2] |
| $CH_2CO.Y_DRGDCGGC_{Acm}GC_{Acm}$amide | 1171 | 99[2] | 13.5[2] |
| $CH_2CO.Y_D.Apc.GDCGGGC_{Acm}GC_{Acm}$amide | 1233 | 100[4] | 17.1, 18.1[2] |
| $CH_2CO.Y_DKGDCGGGC_{Acm}GC_{Acm}$amide | 1200 | 96[4] | 15.8, 16.1[2] |
| Pic.$GC_{Acm}$GGCNP.Apc.GDC | 1217 | 70[2] | 6.6-13.7[2] |
| *Ac*.$C_{Acm}GC_{Acm}$GGCNP.Apc.GDC | 1327 | 98[2] | 13.0-15.5[2] |
| *Ac*.CNP.Apc.GDC | 810 | 99[1] | 12.9[2] |
| $C_{Acm}GC_{Acm}$GGRGDS | 953 | 100[2] | 8.6[1] |
| $C_{Acm}GC_{Acm}$GGRGDGGRGDS | 1396 | 100[1] | 12.6[1] |
| $C_{Acm}GC_{Acm}$GGRGDGGRGDGGRGDS | 1838 | 100[2] | 10.0, 10.8[1] |
| $C_{Acm}GC_{Acm}$RRRRRRRRRGDV | 2100 | 100[2] | 2.4[3]*** |

TABLE I (cont'd.)

| Peptides | FABMS MH$^+$ | Radiochemical Yield(%)* | HPLC $R_T$(min)** |
|---|---|---|---|
| GRGDVKC$_{Acm}$GC$_{Acm}$amide | 1036 | 100[2] | 15.7[2] |
| GRGDVC$_{Acm}$GC$_{Acm}$amide | 907 | 100[2] | 16.1[2] |
| GRGDVRGDFKC$_{Acm}$GC$_{Acm}$amide | 1510 | 97[2] | 16.2, 16.8[2] |
| GRGDVRGDFC$_{Acm}$GC$_{Acm}$amide | 1382 | 94[2] | 16.4[2] |
| (GPRVVERHQSA)$_2$K | 2986 | 99[4] | 16.0[2] |
| CRGDC | 553 | 100[3] | 16.7[2] |
| GRGDGGC | 769 | 98[1] | 13.0, 13.6, 14.7[2] |
| CH$_2$CO.YIGSRCGC$_{Acm}$GC$_{Mob}$ | 1249 | 96[2] | 18.0[1] |
| CYGQQHHLGGAKQAGDV | N.D. | 97[2] | 23.8[3] |
| *acetyl*-RRARGDDLDC$_{Acm}$GC$_{Acm}$.amide | 1520 | 98[2] | 10.8[2] |
| Pic.GC$_{Acm}$GQQHHLGGAKQAGDV | 1838 | 48[2] | 14.8[2] |
| *ma*GGRGDF | 739 | 98[1] | 13.8-14.7[2] |

TABLE I (cont'd.)

| Peptides | FABMS MH$^+$ | Radiochemical Yield(%)* | HPLC $R_T$(min)** |
|---|---|---|---|
| *mmp*GGGRGDF | 767 | 100[3] | 18.4, 19.3[2] |
| GRGDGGGGC | 735 | 100[3] | 14.9, 15.1, 15.4[3] |
| *ma*RGD | 421 | 97[3] | 16.1, 16.9, 17.7[2] |
| *ma*RGDF | 568 | 94[3] | 18.1, 18.7[2] |
| CKRARGDDMDDYC | 1548 | 97[3] | 16.7[2] |
| (Pic.SC$_{Acm}$SYNRGDSTC)$_3$-TSEA | 4489 | 99[2] | 10.4,11.2[2] |
| C$_{Acm}$GC$_{Acm}$NDGDFEEIPEEYLQ | 2103 | 100[2] | 2.5[1]**** |
| C$_{Acm}$GC$_{Acm}$GGF$_D$PRPGGGGNGDFEEIPEEYL | 2699 | 99[2] | 14.5[3] |
| *ma*GGGF$_D$PRPGGGGNGDFEEIPEEYL | 2426 | 95[2] | 17.4[2] |
| C$_{Acm}$GC$_{Acm}$GGF$_D$PRPGamide | 1092 | 100[5] | 9.6[2] |
| (GPRPC$_{Acm}$GC$_{Acm}$C)$_3$-TSEA | 3189 | 93[2] | 10.0[2] |
| [(GPRP)$_2$K]$_2$KC$_{Acm}$GC$_{Acm}$amide | 2437 | 100[4] | 16.3[2] |
| (CC$_{Acm}$GC$_{Acm}$GGRDGS)$_3$-TSEA | N.D. | 81[2] | 9.9 - 11.1[2] |

TABLE I (cont'd.)

| Peptides | FABMS MH$^+$ | Radiochemical Yield(%)* | HPLC $R_T$(min)** |
|---|---|---|---|
| PSPSPIHPAHHKRDRRQC$_{Acm}$GC$_{Acm}$.amide | 2421 | 94[2] | 13.4[2] |
| (*acetyl*-F$_D$PRPG)$_2$KGGGC.amide | 1613 | 98[3] | 17.4[2] |
| $CH_2CO$.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGF$_D$PRPG.amide | 1845 | 90[4] | 16.6, 16.9[2] |
| $CH_2CO$.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$.GGCamide | 1392 | 99[3] | 11.7[4] |
| *acetyl*-G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide | 1561 | 100[4] | 9.3, 9.8[2] |
| [BAT].Hly.GDP.Hly.GDF.amide | 1209 | 100[3] | 10.8[2] |
| ($CH_2CO$.Y$_D$Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGC.amide)$_2$-BSME | 3020[a] | 98[4] | 9.3[2] |
| $CH_2CO$.Y$_D$Apc.GDCKGC$_{Acm}$GC$_{Acm}$.amide | 1282 | 99[4] | 15.8, 16.1[2] |
| $CH_2CO$.Y$_D$.Apc.GDC.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide | 1669 | 99[4] | 16.2, 16.6[2] |
| G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide | 1519 | 99[4] | 9.3, 9.8[2] |
| G.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide | 1040 | 100[4] | 9.4[2] |
| ($CH_2CO$.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGC.amide)$_3$-TSEA | 4596 | 99[4] | 9.2, 11.6[5] |
| *acetyl*-RGDC.amide | 490 | 94[3] | 8.5[5] |
| (GPRPPPGGC$_{Acm}$GC$_{Acm}$GGC.amide)$_3$-TSEA | 4454[a] | 100[4] | 9.1[5] |
| ($CH_2CO$.Y$_D$Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGC.amide)$_2$-[BAT-BS] | 3409[a] | 98[3] | 10.3[5] |
| Pic.GC$_{Acm}$PSPSPIHPAHHKRDRRQ.amide | 2351 | 94[6] | 11.2[2] |

TABLE I (cont'd.)

| Peptides | FABMS MH$^+$ | Radiochemical Yield(%)* | HPLC $R_T$(min)** |
|---|---|---|---|
| CH$_2$CO.YIGSRCGC$_{Acm}$GC$_{Acm}$.amide | 1199 | 94[2] | 16.8[2] |
| GRGDGGFC$_{Acm}$ | 839 | N.D. | N.D. |
| C$_{Acm}$GGGRGDF | 839 | 99[7] | 15.7-17.3[2] |
| GRGDGGC$_{Acm}$ | 692 | 98[2] | 14.1, 14.4[2] |
| *ma*$_{Bz}$-GGGRGDF | 843 | 100[2] | 16.7[2] |
| *ma*$_{Acm}$-GGGRGDF | 810 | 100[7] | 16.1[2] |
| CH$_2$CO.Y$_{D.Apc}$.GDCGGC$_{Acm}$GC$_{Acm}$GGC$_{NES}$.amide | 1517 | N.D. | N.D. |
| CH$_2$CO.Y$_{D.Apc}$.GDCKGC$_{Acm}$GC$_{Acm}$GGC.amide | 1485 | N.D. | N.D. |
| CRIARGDWNDDYC | 1587 | N.D. | N.D. |
| CKFFARTVCRIARGDWNDDYCTGKSSDC | 3329 | N.D. | N.D. |
| (CH$_2$CO.Y$_{D.Apc}$.GDCGGC$_{Acm}$GC$_{Acm}$GGC.amide)$_2$-BSH | 3062 | 100[4] | 11.5[4] |
| (CH$_2$CO.Y$_{D.Apc}$.GDCKGC$_{Acm}$GC$_{Acm}$GGC.amide)$_3$-BAT-BS | 3552 | N.D. | N.D. |
| CH$_2$CO.Y$_D$RGDCDGGC$_{Acm}$GC$_{Acm}$.amide | 1287 | 96[2] | 11.6, 11.9[2] |
| [BAT]G.Apc.GDV.Apc.GDFK.amide | 1432 | 96[4] | 17.5[2] |
| (CH$_2$CO.Y$_D$Apc.GDCKGC$_{Acm}$GC$_{Acm}$GGC.amide)$_2$-BSME | 3163[a] | 98[4] | 9.6[5] |

* Superscripts refer to the following labeling conditions:

1. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 7.4) and labeled at room temperature.
2. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 7.4) and labeled at 100°C.
3. The peptide is dissolved in water and labeled at room temperature.
4. The peptide is dissolved in water and labeled at 100°C.
5. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 6.0) and labeled at 100°C.
6. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 5.0) and labeled at room temperature.
7. The peptide is dissolved in a 50:50 mixture of ethanol/water and labeled at 100°C.

** HPLC methods (indicated by superscript after $R_T$):

| | | |
|---|---|---|
| general: | solvent A | $= 0.1\%\ CF_3COOH/H_2O$ |
| | solvent $B_{70}$ | $= 0.1\%\ CF_3COOH/70\%\ CH_3CN/H_2O$ |
| | solvent $B_{90}$ | $= 0.1\%\ CF_3COOH/90\%\ CH_3CN/H_2O$ |
| | solvent flow rate = 1 mL/min | |

Vydak column = Vydak 218TP54 RP-18, 5μ x 220mm x 4.6mm analytical column with guard column
Brownlee column = Brownlee Spheri-5 RP-18, 5μ x 220mm x 4.6mm column
Waters column = Waters Delta-Pak C18, 5μ x 150mm x 3.9mm column

| | | |
|---|---|---|
| Method 1: | Brownlee column | 100% A to 100% $B_{70}$ in 10 min |
| Method 2: | Vydak column | 100% A to 100% $B_{90}$ in 10 min |
| Method 3: | Vydak column | 100% A to 100% $B_{70}$ in 10 min |
| Method 4: | Waters column | 100% A to 100% $B_{90}$ in 20 min |
| Method 5: | Waters column | 100% A to 100% $B_{90}$ in 10 min |

*** Confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

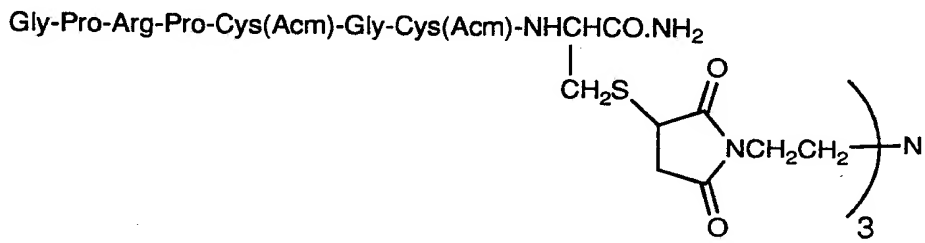
**** Confirmed by binding the peptide to an affinity column

Single-letter abbreviations for amino acids can be found in G. Zubay, *Biochemistry*

(2d. ed.), 1988 (MacMillen Publishing: New York) p.33; underlining indicates the formation of a thiol linkage between the linked amino acids of derivative groups; peptides are linked to BSH, ETAC, BSME, TSEA or [BAT-BS] linkers *via* the free thiol moiety of the unprotected cysteine residue (C) in each peptide; Ac = acetyl; Bz = benzoyl; Pic = picolinoyl (pyridine-2-carbonyl); Acm = acetamidomethyl; Mob = 4-methoxybenzyl; Apc = L-[S-(3-aminopropyl)cysteine; Hly = homolysine; $F_D$ = D-phenylalanine; $Y_D$ = D-tyrosine; ma = 2-mercaptoacetic acid; mmp = 2-mercapto-2-methylpropionic acid; BAT = $N^6$,$N^9$-*bis*(2-mercapto-2-methylpropyl)-6,9-diazanonanoic acid; ETAC = 4-(O-$CH_2$CO-Gly-Gly-Cys.amide)acetophenone; BAT-BS = N-[2-N_,N_-*bis*(2-succinimidoethyl) aminoethyl]-$N^6$,$N^9$-*bis*(2-mercapto-2-methylpropyl)-6,9-diazanonanamide; BSME = *bis*-succinimidomethylether; TSEA = *tris*-(2-succinimidoethyl)amine; NES = N-ethylsuccinimide; BSH = 1,6-*bis*-succinimidohexane

[a] = confirmed by electrospray mass spectrometry [ESMS]

B.

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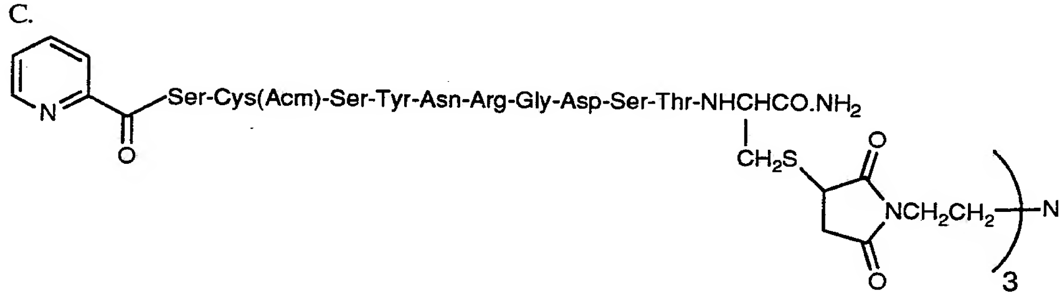


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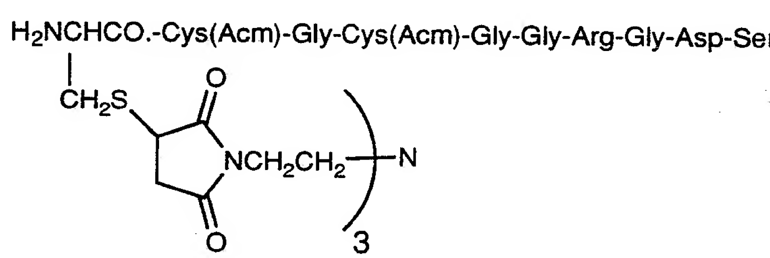

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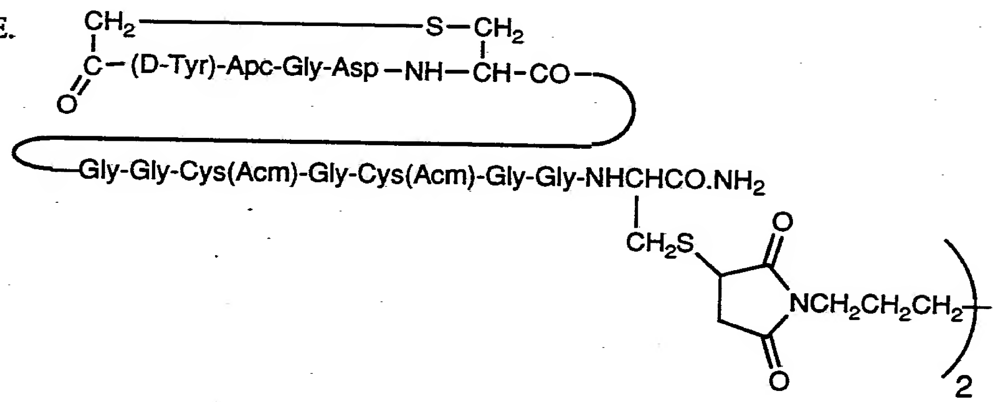

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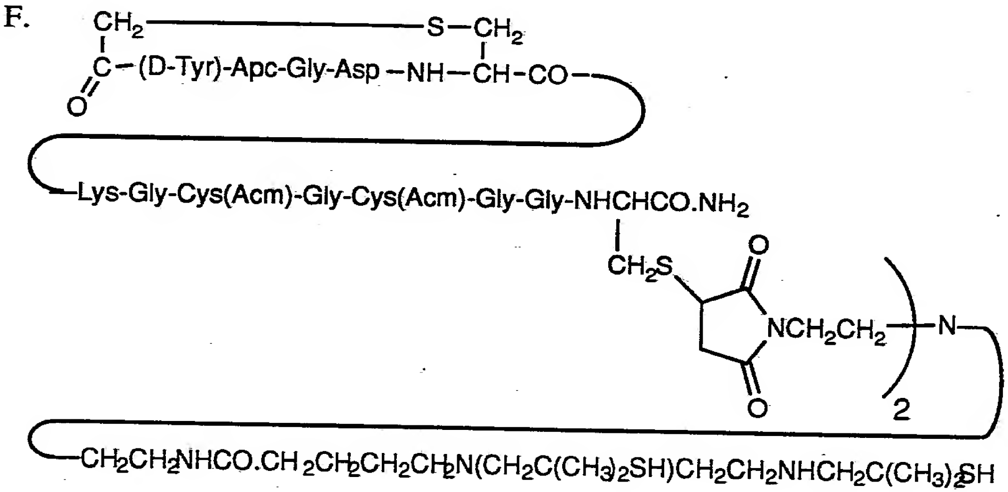
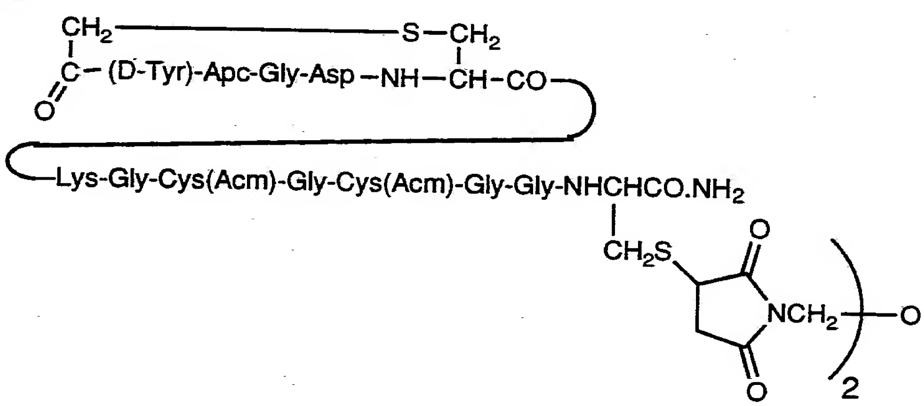


G.



## EXAMPLE 3

### Platelet Aggregation Inhibition Assays

Platelet aggregation studies were performed essentially as described by Zucker (1989, Methods in Enzymol. 169: 117-133). Briefly, platelet aggregation was assayed with or without putative platelet aggregation inhibitory compounds using fresh human platelet-rich plasma, comprising 300,000 platelets per microlitre. Platelet aggregation was induced by the addition of a solution of adenosine diphosphate to a final concentration of 10 to 15 micromolar, and the extent of platelet aggregation monitored using a Bio/Data aggregometer (Bio/Data Corp., Horsham, PA). The concentrations of platelet aggregation inhibitory compounds used were varied from 0.1 to 500 µg/mL. The concentration of inhibitor that reduced the extent of platelet aggregation by 50% (defined as the $IC_{50}$) was determined from plots of inhibitor concentration *versus* extent of platelet aggregation. An inhibition curve for peptide RGDS was determined for each batch of platelets tested as a positive control.

The results of these experiments are shown in Table I. In Table I, the compounds tested are as follows:

P97 = **GRGDVRGD**FKC$_{Acm}$GC$_{Acm}$amide

P32 = C$_{Acm}$GC$_{Acm}$RRRRRRRRRGDV

P143 = CH$_2$CO-Y$_D$**RGDC**GGC$_{Acm}$GC$_{Acm}$amide

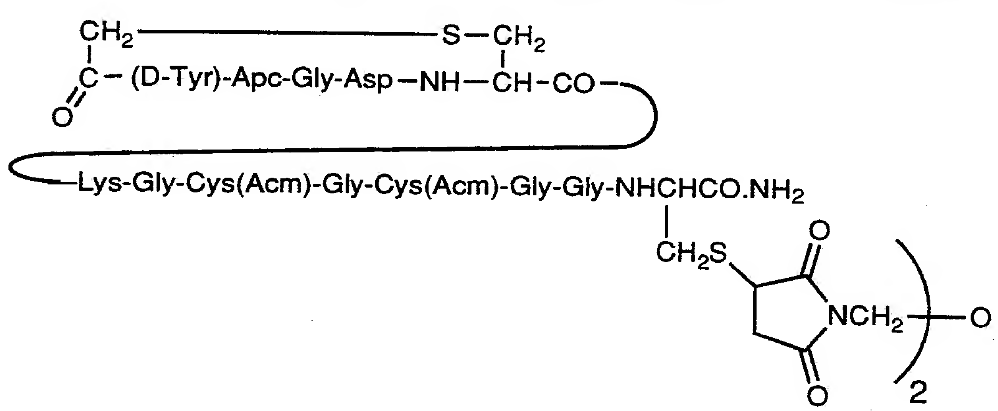
P245 = CH$_2$CO-Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGF$_D$PRPGamide

P98 = **GRDGVRGD**FC$_{Acm}$GC$_{Acm}$amide

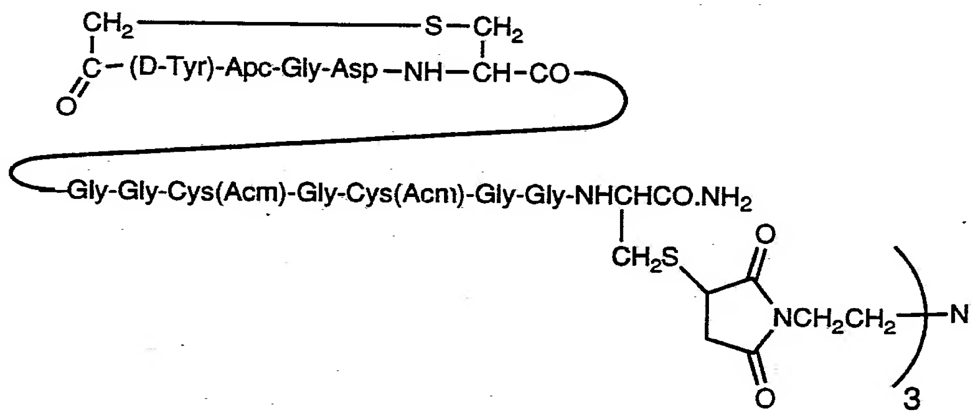
P81 = CH$_2$CO-Y$_D$**RGDC**C$_{Acm}$GC$_{Acm}$amide

P154 = CH$_2$CO-Y$_D$**ApcGDC**GGGC$_{Acm}$GC$_{Acm}$amide

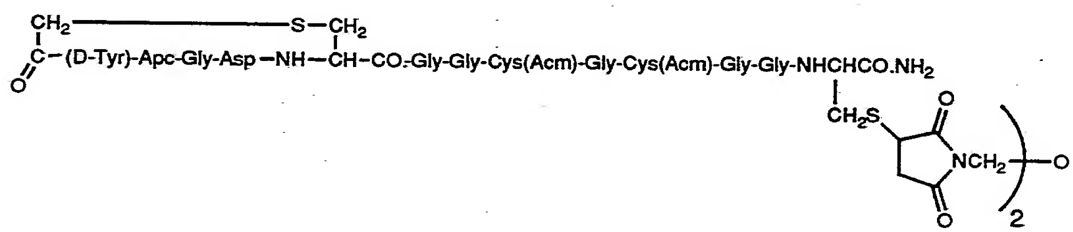
P381 = (CH$_2$CO-Y$_D$**ApcGDC**KGC$_{Acm}$GC$_{Acm}$GGC-amide)$_2$-BSME



P317 = (CH$_2$CO-Y$_D$ApcGDCGGC$_{Acm}$GC$_{Acm}$GGC-amide)$_3$-TSEA

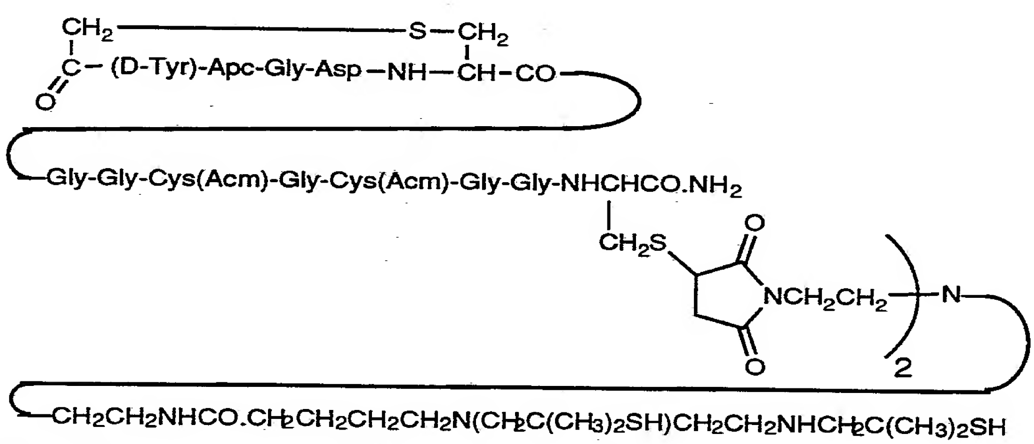


P280 = (CH$_2$CO-Y$_D$ApcGDCGGC$_{Acm}$GC$_{Acm}$GGC-amide)$_2$-BSME



P357 = (CH$_2$CO-Y$_D$ApcGDCGGC$_{Acm}$GC$_{Acm}$GGC-amide)$_2$-[BAT-BS]

P357



(Abbreviations are as found in the Legend of Table I).

These results demonstrate that peptide reagents of this invention bind with high affinity to activated platelets, in many cases with higher affinity than the naturally-occurring sequence RGDS.

**TABLE II**

| Peptides | IC50(µM)* |
|---|---|
| P317 | 0.036 |
| P381 | 0.035 |
| P357 | 0.081 |
| P280 | 0.090 |
| P154 | 0.3 |
| P245 | 0.5 |
| P143 | 1.3 |
| P97 | 8 |
| P98 | 15 |
| P81 | 25 |
| P32 | 26 |
| RGDS | 150-250 |

*concentration of reagent that inhibits by 50% the aggregation of human platelets in platelet-rich plasma inducedto aggregate by the addition of adenosine diphosphate (ADP).

## EXAMPLE 4
## *In Vivo* Imaging of Deep Vein Thrombosis using a Tc-99m Labeled Peptide in a Canine Model

Mongrel dogs (25-35lb., fasted overnight) were sedated with a combination of ketamine and aceprozamine intramuscularly and then anesthetized with sodium pentabarbital intravenously. In each animal, an 18-gauge angiocath was inserted in the distal half of the right femoral vein and an 8mm Dacron®-entwined stainless steel embolization coil (Cook Co., Bloomington IN) was placed in the femoral vein at approximately mid-femur. The catheter was removed, the wound sutured and the placement of the coil documented by X-ray. The animals were then allowed to recover overnight.

One day following coil placement, each animal was re-anesthetized, intravenous saline drips placed in each foreleg and a urinary bladder catheter inserted to collect urine. The animal was placed supine under a gamma camera which was equipped with a low-energy, all purpose collimator and photopeaked for Tc-99m.

Tc-99m labeled peptide [185-370 mBq (5-10 mCi) Tc-99m] was injected sequentially into one foreleg intravenous line at its point of insertion. The second line was maintained for blood collection.

Gamma camera imaging was started simultaneously with injection. Anterior images over the heart were acquired as a dynamic study (10 sec image acquisitions) over the first 10 min, and then as static images at 1, 2, 3 and 4h post-injection. Anterior images over the legs were acquired for 500,000 counts or 20 min (whichever was shorter), at approximately 10-20 min, and at approximately 1, 2, 3 and 4h post-injection. Leg images were collected with a lead shield placed over the bladder.

Following the final image, each animal was deeply anesthetized with pentobarbital. Two blood samples were collected on a cardiac puncture using a heparinized syringe followed by a euthanasing dose of saturated potassium chloride solution administered by intercardiac or bolus intravenous injection. The femoral vein containing the thrombus, a similar section of vein of the contralateral (control) leg, sections of the vessel proximal to the thrombus and samples of thigh muscle were then carefully dissected out. The thrombus, coil and coil Dacron fibres were then dissected free of the vessel. The thrombus, saline-washed vessel samples, coil and coil Dacron fibres were separated, and each sample was placed in a pre-weighed test tube. The samples were weighed and counted in a gamma well counter in the Tc-99m channel, along with known fractions of the injected doses.

Fresh thrombus weight, percent injected dose (%ID)/g in the thrombus and

blood obtained just prior to euthanasia and thrombus/blood and thrombus/muscle ratios were determined. From the computer-stored images, thrombus/background ratios were determined by analysis of the counts/pixel measured in regions-of-interest (ROI) drawn over the thrombus and adjacent muscle. Tissue data from these experiments are shown in the following Table. Scintigraphic images showing the locaiton of venous thrombi *in vivo* detected using Tc-99m labeled peptide are shown in Figure 1.

These results demonstrate that deep vein thrombi can be rapidly and efficiently located *in vivo* using Tc-99m labeled reagents of the invention. Localization was clearly established within 1h post-injection and persisted, with increasing contrast and focal definition, over nearly 4h post-injection.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

**TABLE III**

| Peptide | Thrombus/ Background | %ID/g Thrombus | %ID/g Blood | Thrombus/ Blood | Thrombus/ Muscle |
|---|---|---|---|---|---|
| P317 (n=4)[a] | N.D. | 0.0035 | 0.0011 | 3.8 ± 2.2 | 16 ± 10 |
| P280 (n=6) | 2.3 ± 0.4 | 0.0059 | 0.0012 | 4.4 ± 1.8 | 11 ± 7 |
| P357 (n=9) | N.D. | 0.019 | 0.0028 | 11 ± 7 | 21 ± 14 |

Values shown are the average ± the standard deviation from the mean;

[[a] n=number of experiments performed with this peptide]

What is claimed is:

1. A reagent for preparing a scintigraphic imaging agent for imaging a thrombus within a mammalian body comprising a specific binding compound capable of binding to at least one component of a thrombus, covalently linked to a technetium-99m binding moiety, wherein the technetium-99m binding moiety has the formula:

$$C(pgp)^{S}\text{-}(aa)\text{-}C(pgp)^{S}$$

wherein $C(pgp)^{S}$ is a cysteine having a protected thiol group and (aa) is an amino acid.

2. The reagent of Claim 1 that is radiolabeled with technetium-99m.

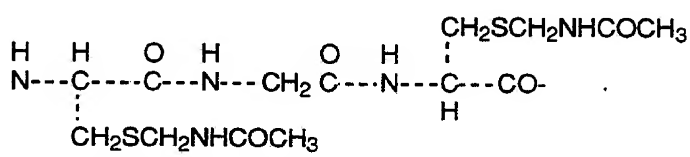
3. The reagent of Claim 1 wherein the specific binding compound and $C(pgp)^{S}\text{-}(aa)\text{-}C(pgp)^{S}$ are covalently linked through from about one to about 20 amino acids.

4. The reagent of Claim 1 wherein the protected cysteine has a protecting group of the formula

$$-CH_2\text{-}NH\text{-}CO\text{-}R$$

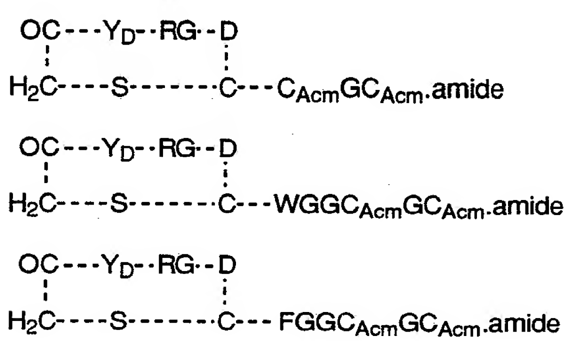
wherein R is a lower alkyl having 1 to 6 carbon atoms, 2-,3-,4-pyridyl, phenyl, or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl.

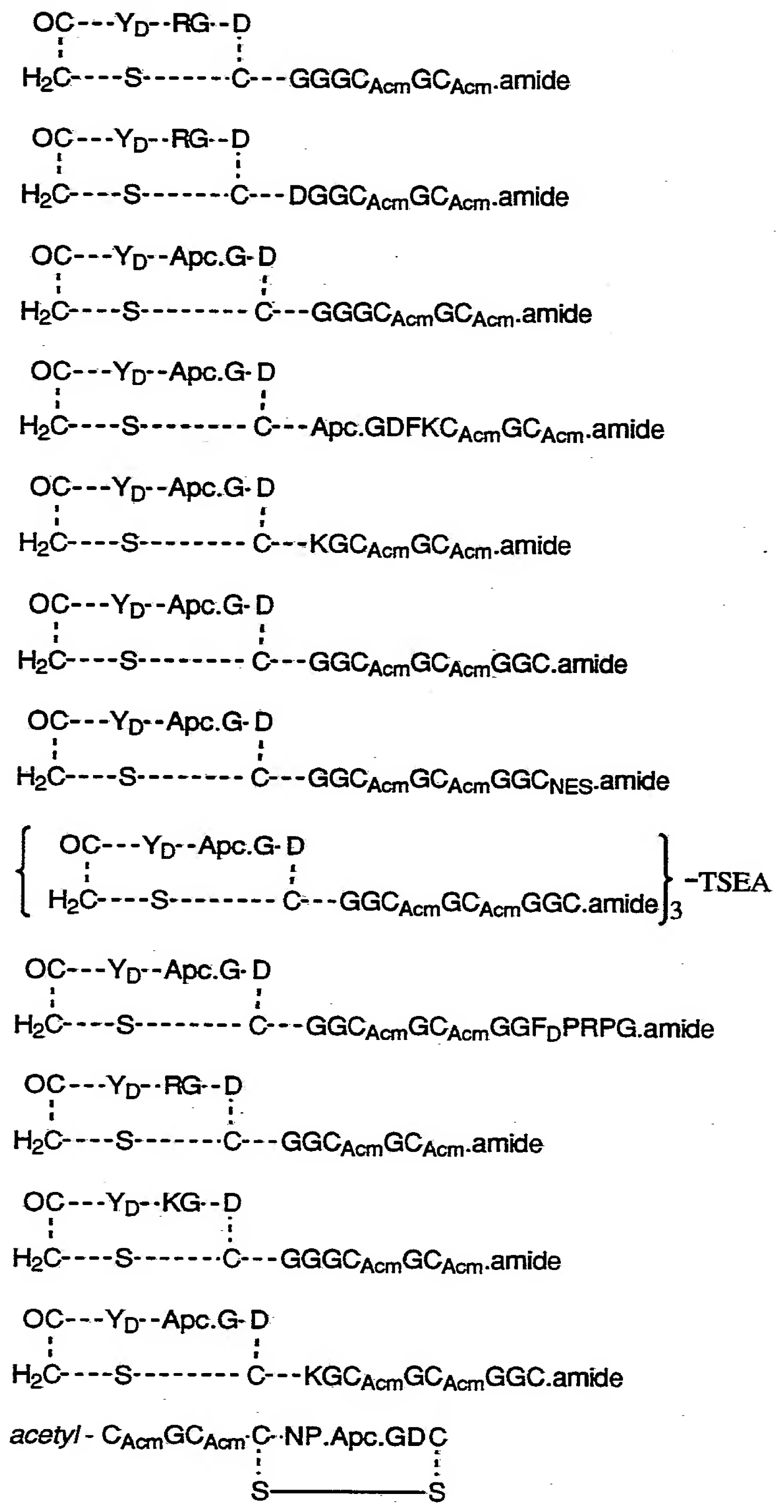
5. The reagent of Claim 1 wherein $C(pgp)^{S}\text{-}(aa)\text{-}C(pgp)^{S}$ has the formula:




6. The reagent of Claim 1 wherein the specific binding compound is a peptide comprising 4 to 100 amino acids.

7. The reagent of Claim 6 having the formula:





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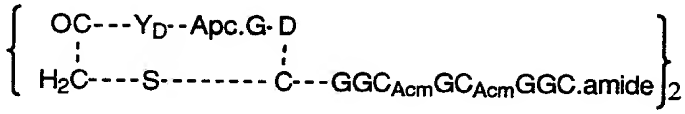
H2C----S-------C---GGGCAcmGCAcm.amide
OC---YD--RG--D
H2C----S-------C---DGGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S-------- C---GGGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S-------- C---Apc.GDFKCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S-------- C---KGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S-------- C---GGCAcmGCAcmGGC.amide
OC---YD--Apc.G-D
H2C----S-------- C---GGCAcmGCAcmGGCNES.amide
OC---YD--Apc.G-D
H2C----S-------- C---GGCAcmGCAcmGGC.amide
3
-TSEA
OC---YD--Apc.G-D
H2C----S-------- C---GGCAcmGCAcmGGFDPRPG.amide
OC---YD--RG--D
H2C----S-------C---GGCAcmGCAcm.amide
OC---YD--KG--D
H2C----S-------C---GGGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S-------- C---KGCAcmGCAcmGGC.amide
acetyl - CAcmGCAcm-C-NP.Apc.GDC
S S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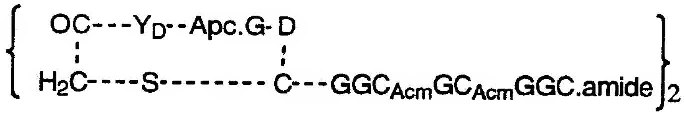
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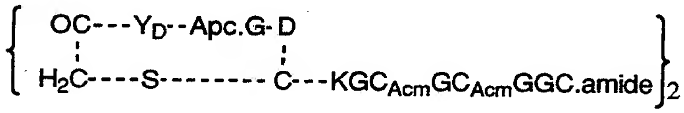
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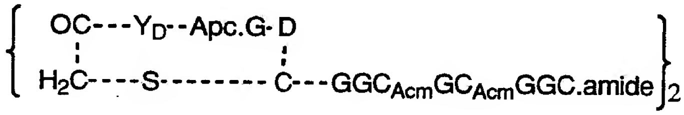
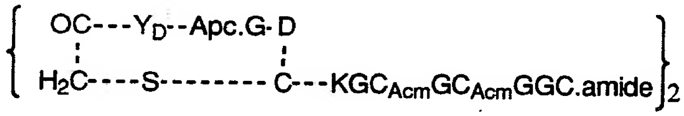

-[BAT-BS]


-[BAT-BS]

C$_{Acm}$GC$_{Acm}$GGRGDS
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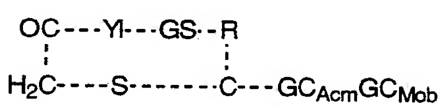
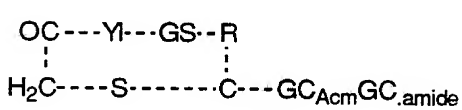
C$_{Acm}$GC$_{Acm}$RRRRRRRRRGDV
GRGDVKC$_{Acm}$GC$_{Acm}$.amide
GRGDVC$_{Acm}$GC$_{Acm}$.amide
GRGDVRGDFKC$_{Acm}$GC$_{Acm}$.amide
GRGDVRGDFC$_{Acm}$GC$_{Acm}$.amide
*acetyl*-G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide
G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide
G.Apc.GDVKC$_{Acm}$GC$_{Acm}$.amide
(CC$_{Acm}$GC$_{Acm}$GGRGDS)$_3$-TSEA
C$_{Acm}$GC$_{Acm}$NDGDFEEIPEEYLQ
C$_{Acm}$GC$_{Acm}$GGF$_D$PRPGGGGNGDFEEIPEEYL
C$_{Acm}$GC$_{Acm}$GGF$_D$PRPGamide
[(GPRP)$_2$K]$_2$KC$_{Acm}$GC$_{Acm}$.amide
(GPRVVERHQSA)$_2$KC$_{Acm}$GC$_{Acm}$.amide
(GPRPC$_{Acm}$GC$_{Acm}$C)$_3$-TSEA
[GPRPPPGGC$_{Acm}$GC$_{Acm}$GGC]$_3$-TSEA





*acetyl*-RRARGDDLDC$_{Acm}$GC$_{Acm}$.amide
PSPSPIHPAHHKRDRRQC$_{Acm}$GC$_{Acm}$.amide

8. A complex formed by reacting the reagent of Claim 1 with technetium-99m in the presence of a reducing agent.

9. The complex of Claim 8, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

10. A complex formed by labeling the reagent of Claim 1 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

11. A kit for preparing a radiopharmaceutical preparation, said kit comprising a sealed vial containing a predetermined quantity of the reagent of Claim 1 and a sufficient amount of reducing agent to label the reagent with technetium-99m.

12. A method for labeling a reagent according to Claim 1 comprising reacting the reagent with technetium-99m in the presence of a reducing agent.

13. The method of Claim 12, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

14. A method for imaging a thrombus within a mammalian body comprising administering an effective diagnostic amount of the reagent of Claim 2 and detecting the technetium-99m localized at the site of a thrombus.

15. The reagent according to Claim 1 wherein the specific-binding peptide is chemically synthesized *in vitro*.

16. The reagent according to Claim 15 wherein the specific-binding peptide is synthesized by solid phase peptide synthesis.

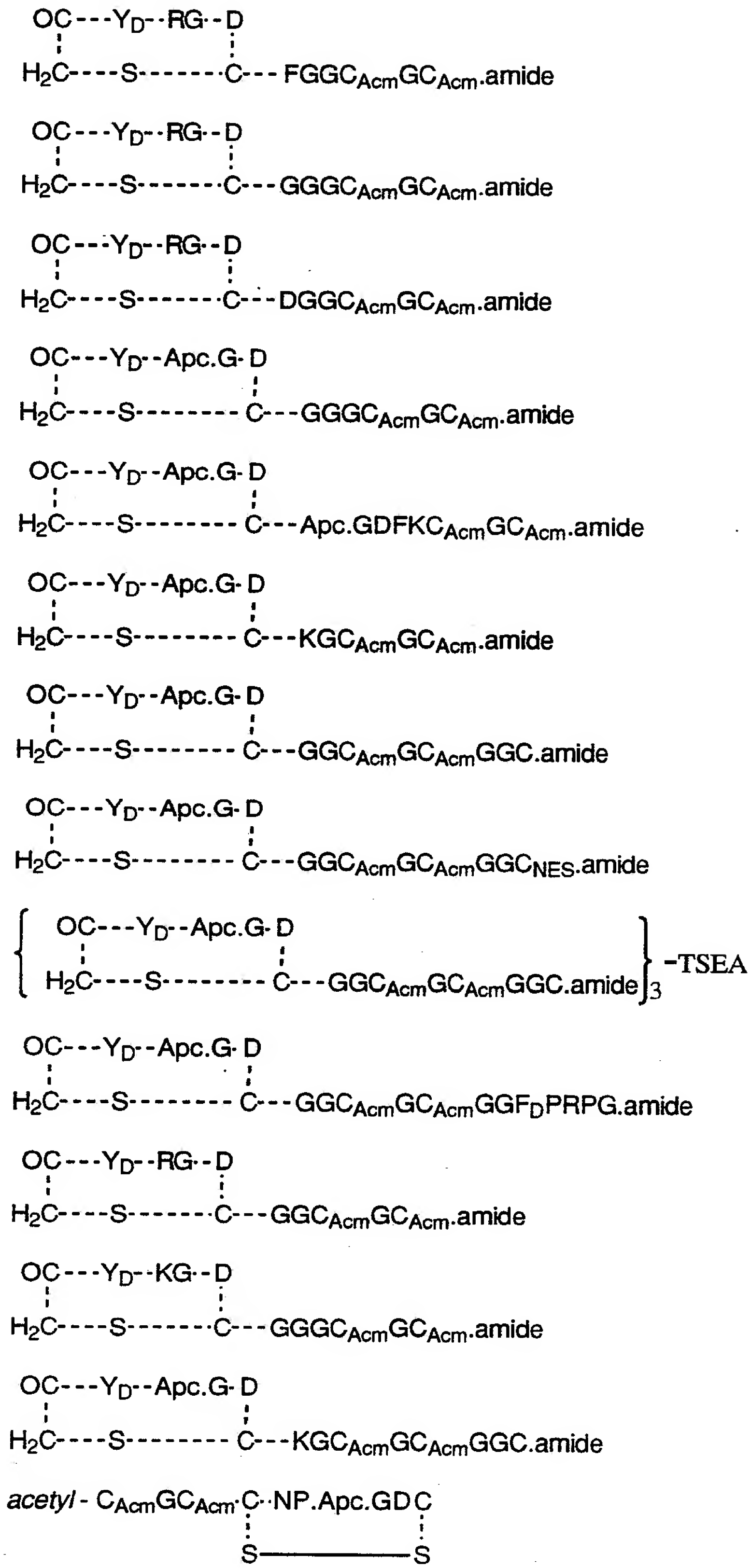
17. The reagent according to Claim 15 wherein the radiolabel-binding moiety is covalently linked to the specific-binding peptide during *in vitro* chemical synthesis.

18. The reagent according to Claim 17 wherein the radiolabel-binding moiety is covalently linked to the specific-binding peptide during solid phase peptide synthesis.

19. A composition of matter comprising a reagent having the formula:

```
OC---YD--RG--D
:            :
H2C----S------C---CAcmGCAcm.amide

OC---YD--RG--D
:            :
H2C----S------C---WGGCAcmGCAcm.amide
```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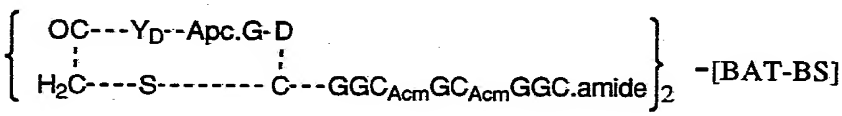
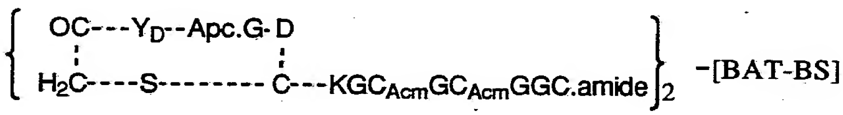
H2C----S-------C---DGGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S--------C---GGGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S--------C---Apc.GDFKCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S--------C---KGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S--------C---GGCAcmGCAcmGGC.amide
OC---YD--Apc.G-D
H2C----S--------C---GGCAcmGCAcmGGCNES.amide
OC---YD--Apc.G-D
H2C----S--------C---GGCAcmGCAcmGGC.amide
3
-TSEA
OC---YD--Apc.G-D
H2C----S--------C---GGCAcmGCAcmGGFDPRPG.amide
OC---YD--RG--D
H2C----S-------C---GGCAcmGCAcm.amide
OC---YD--KG--D
H2C----S-------C---GGGCAcmGCAcm.amide
OC---YD--Apc.G-D
H2C----S--------C---KGCAcmGCAcmGGC.amide
acetyl - CAcmGCAcm-C--NP.Apc.GDC
S S




$C_{Acm}GC_{Acm}$GGRGDS
$C_{Acm}GC_{Acm}$GGRGDGGRGDS
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$C_{Acm}GC_{Acm}$RRRRRRRRRGDV
GRGDVK$C_{Acm}GC_{Acm}$.amide
GRGDV$C_{Acm}GC_{Acm}$.amide
GRGDVRGDFK$C_{Acm}GC_{Acm}$.amide
GRGDVRGDF$C_{Acm}GC_{Acm}$.amide
*acetyl*-G.Apc.GDV.Apc.GDFK$C_{Acm}GC_{Acm}$.amide
G.Apc.GDV.Apc.GDFK$C_{Acm}GC_{Acm}$.amide
G.Apc.GDVK$C_{Acm}GC_{Acm}$.amide
(C$C_{Acm}GC_{Acm}$GGRGDS)$_3$-TSEA
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(GPRVVERHQSA)$_2$K$C_{Acm}GC_{Acm}$.amide
(GPRP$C_{Acm}GC_{Acm}$C)$_3$-TSEA

[GPRPPPGG$C_{Acm}GC_{Acm}$GGC]$_3$-TSEA

```
OC---Yl---GS--R
 :            :
H2C----S-------C---GC(Acm)GC(Mob)

OC---Yl---GS--R
 :            :
H2C----S-------C---GC(Acm)GC.amide
```

*acetyl*-RRARGDDLD$C_{Acm}$G$C_{Acm}$.amide

PSPSPIHPAHHKRDRRQ$C_{Acm}$G$C_{Acm}$.amide

20. The reagent of Claim 1 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

21. The reagent of Claim 20 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N_*,*N_*-*bis*(2-succinimido-ethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine, *bis*-succinimidohexane, 4-(O-$CH_2$CO-Gly-Gly-Cys.amide)acetophenone or a derivative thereof.

22. A reagent for preparing a thrombus imaging agent for imaging a thrombus within a mammalian body comprising a specific binding compound capable of binding to at least one component of a thrombus, covalently linked to a technetium-99m binding moiety, wherein the technetium-99m binding moiety has the formula:

$$A\text{-}CZ(B)\text{-}[C(R^1R^2)]_n\text{-}X$$

wherein A is H, HOOC, $H_2$NOC, (peptide)-NHOC, (peptide)-OOC or $R^4$;
B is H, SH, -NH$R^3$, -N($R^3$)-(peptide), or $R^4$;
X is H, SH, -NH$R^3$, -N($R^3$)-(peptide) or $R^4$;
Z is H or $R^4$;
$R^1$, $R^2$, $R^3$ and $R^4$ are independently H or lower straight or branched chain or cyclic alkyl;
n is 0, 1 or 2;

and

where B is -NH$R^3$ or -N($R^3$)-(peptide), X is SH, and n is 1 or 2;
where X is -NH$R^3$ or -N($R^3$)-(peptide), B is SH, and n is 1 or 2;
where B is H or $R^4$, A is HOOC, $H_2$NOC, (peptide)-NHOC, (peptide)-OOC, X is SH, and n is 0 or 1;

where A is H or $R^4$, then where B is SH, X is $-NHR^3$ or $-N(R^3)$-(peptide) and where X is SH, B is $-NHR^3$ or $-N(R^3)$-(peptide);
where X is H or $R^4$, A is HOOC, $H_2NOC$, (peptide)-NHOC, (peptide)-OOC and B is SH;
where Z is methyl, X is methyl, A is HOOC, $H_2NOC$, (peptide)-NHOC, (peptide)-OOC, B is SH and n is 0;
where B is SH and X is SH, n is not 0;
and wherein the thiol moiety is in the reduced form.

23. The reagent of Claim 1 wherein the specific binding compound is a peptide comprising 4 to 100 amino acids.

24. The reagent of Claim 22 that is radiolabled with technetium-99m.

25. The reagent according to Claim 22 wherein the specific-binding compound and the technetium-99m binding moiety are covalently linked through from about one to about 20 amino acids.

26. A complex formed by reacting the reagent according to Claim 22 with technetium-99m in the presence of a reducing agent.

27. The complex of Claim 26, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

28. A complex formed by labeling the reagent according to Claim 22 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

29. A composition of matter comprising the reagent according to Claim 22 and a stannous ion.

30. A kit for preparing a radiopharmaceutical preparation, said kit comprising a sealed vial containing a predetermined quantity of a reagent according to Claim 22 and a sufficient amount of reducing agent to label said reagent with technetium-99m.

31. A method for labeling a reagent according to Claim 22 comprising reacting the reagent with technetium-99m in the presence of a reducing agent.

32. The method of Claim 31, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

33. A method for imaging a thrombus within a mammalian body comprising administering an effective diagnostic amount of a reagent according to Claim 24 and detecting the technetium-99m localized at the thrombus.

34. The reagent according to Claim 22 wherein the specific-binding peptide is chemically synthesized *in vitro*.

35. The specific-binding peptide according to Claim 34 wherein the peptide is synthesized by solid phase peptide synthesis.

36. The reagent according to Claim 34 wherein the technetium-99m binding moiety is covalently linked to the peptide during *in vitro* chemical synthesis.

37. The reagent according to Claim 36 wherein the technetium-99m binding moiety is covalently linked to the peptide during solid phase peptide synthesis.

38. The reagent of Claim 22 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

39. The reagent of Claim 38 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N*_,*N*_-*bis*(2-succinimido-ethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine, *bis*-succinimidohexane, 4-(O-$CH_2$CO-Gly-Gly-Cys.amide)acetophenone or a derivative thereof.

40. The reagent of Claim 22 having the formula:

CKRARGDDMDDYC
*mmp*-GGGRGDF
*acetyl*-RGDC.amide
CRGDC
GRGDFGGC$_{Acm}$
*ma*$_{Bz}$-GGRGDF
C$_{Acm}$GGGRGDF
GRGDGGGGC
GRGDGGC$_{Acm}$
*ma*-GGRGDF
*ma*$_{Acm}$-GGGRGDF
*ma*-RGDF
*ma*-RGD
*acetyl*-CNP.Apc.GDC
CRIARGDWNDDYC
CKFFARTVCRIARGDWNDDYCTGKSSDC
*ma*-GGGGF$_D$PRPGGGGNGDFEEIPEEYL

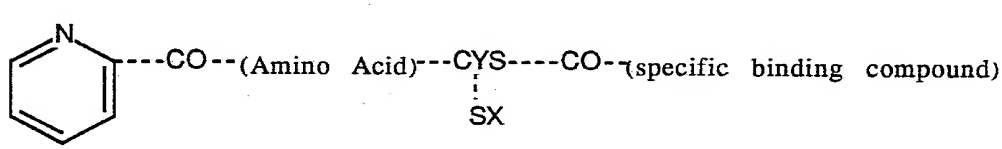
(*acetyl*-F$_{\mathrm{D}}$PRPG)$_2$KGGGC.amide
CYGQQHHLGGAKQAGDV

41. A composition of matter comprising a reagent having the formula:
CKRARGDDMDDYC
*mmp*-GGGRGDF
*acetyl*-RGDC.amide
CRGDC
GRGDFGGC$_{\mathrm{Acm}}$
*ma*$_{Bz}$-GGRGDF
C$_{\mathrm{Acm}}$GGGRGDF
GRGDGGGGC
GRGDGGC$_{\mathrm{Acm}}$
*ma*-GGRGDF
*ma*$_{Acm}$-GGGRGDF
*ma*-RGDF
*ma*-RGD
*acetyl*-CNP.Apc.GDC
CRIARGDWNDDYC
CKFFARTVCRIARGDWNDDYCTGKSSDC
*ma*-GGGGF$_{\mathrm{D}}$PRPGGGGNGDFEEIPEEYL
(*acetyl*-F$_{\mathrm{D}}$PRPG)$_2$KGGGC.amide
CYGQQHHLGGAKQAGDV

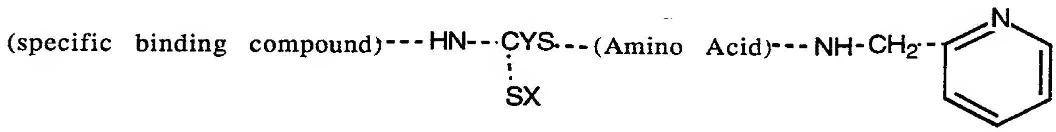
42. A reagent for preparing a thrombus imaging agent for imaging a thrombus within a mammalian body comprising a specific binding compound capable of binding to at least one component of a thrombus, covalently linked to a technetium-99m binding moiety, wherein the technetium-99m binding moiety forms a neutral complex with technetium-99m.

43. The reagent of Claim 42 wherein the technetium-99m binding moiety has a formula selected from the group consisting of:

III.

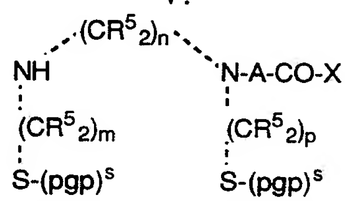


IV.



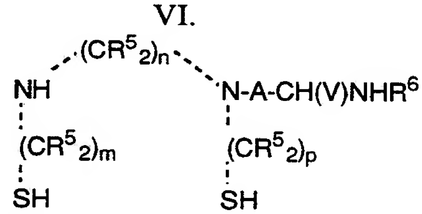

wherein $X = H$ or a protecting group;
(amino acid) = any amino acid;

V.




wherein each $R^5$ is independently H, $CH_3$ or $C_2H_5$;
each $(pgp)^S$ is independently a thiol protecting group or H;
m, n and p are independently 2 or 3;
A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;

VI.




wherein each $R^5$ is independently H, lower alkyl having 1 to 6 carbon atoms, phenyl, or phenyl substituted with lower alkyl or lower alkoxy;
m, n and p are independently 1 or 2;
A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;
V = H or -CO-peptide;
$R^6$ = H or peptide;

and wherein when V = H, $R^6$ = peptide and when $R^6$ = H, V = -CO-peptide; and wherein the technetium-99m binding moiety forms a complex with technetium-99m and the complex of the radiolabel-binding moiety and technetium-99m is electrically neutral.

44. The reagent of Claim 43 wherein the specific binding compound is a peptide comprising 4 to 100 amino acids.

45. The reagent of Claim 43 that is radiolabled with technetium-99m.

46. The reagent according to Claim 43 wherein the specific-binding compound and the technetium-99m binding moiety are covalently linked through about one to about 20 amino acids.

47. A complex formed by reacting the reagent of Claim 43 with technetium-99m in the presence of a reducing agent.

48. The complex of Claim 47, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

49. A complex formed by labeling the reagent of Claim 43 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

50. A kit for preparing a radiopharmaceutical preparation, said kit comprising a sealed vial containing a predetermined quantity of the reagent of Claim 43 and a sufficient amount of reducing agent to label the reagent with technetium-99m.

51. A method for imaging a thrombus within a mammalian body comprising administering an effective diagnostic amount of the reagent of Claim 45 and detecting the technetium-99m localized at the thrombus.

52. The reagent according to Claim 43 wherein the specific-binding peptide is chemically synthesized *in vitro*.

53. The specific-binding peptide according to Claim 52 wherein the peptide is synthesized by solid phase peptide synthesis.

54. The reagent according to Claim 52 wherein the radiolabel-binding moiety is covalently linked to the specific-binding peptide during *in vitro* chemical synthesis.

55. The reagent according to Claim 54 wherein the radiolabel-binding moiety is covalently linked to the specific-binding peptide during solid phase peptide synthesis.

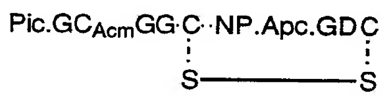
56. The reagent of Claim 43 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

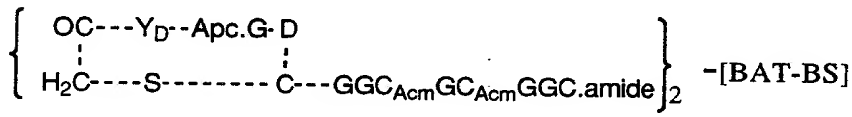
57. The reagent of Claim 56 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N*_,*N*_-*bis*(2-

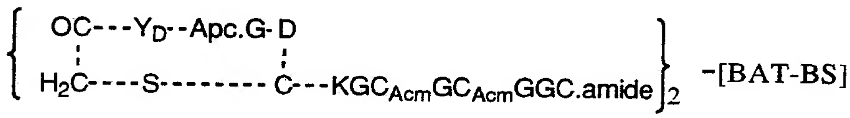
succinimido-ethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine, *bis*-succinimidohexane, 4-(O-$CH_2$CO-Gly-Gly-Cys.amide)acetophenone or a derivative thereof.

58. The reagent of Claim 43 having the formula:

Pic.GC$_{Acm}$PSPSPIHPAHHKRDRRQ.amide
Pic.GC$_{Acm}$GQQHHLGGAKQAGDV
Pic.GC$_{Acm}$GQQHHLGGAKQAGDV
[Pic.SC$_{Acm}$SYNRGDSTC.amide]$_3$-TSEA
[BAT].Hly.GDP.Hly.GDF.amide
[BAT]G.Apc.GDV.Apc.GDFK.amide

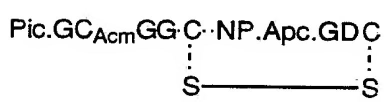


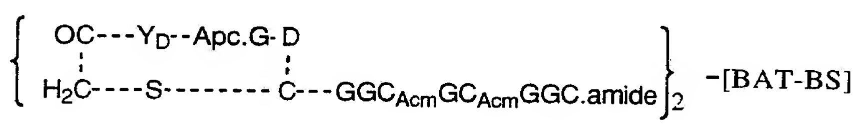



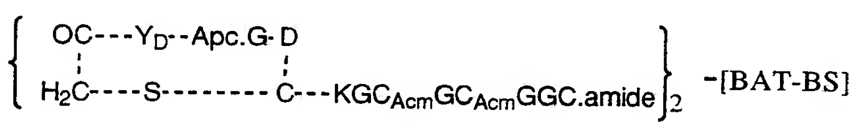




59. A composition of matter comprising a reagent having the formula:

Pic.GC$_{Acm}$PSPSPIHPAHHKRDRRQ.amide
Pic.GC$_{Acm}$GQQHHLGGAKQAGDV
[Pic.SC$_{Acm}$SYNRGDSTC.amide]$_3$-TSEA
[BAT].Hly.GDP.Hly.GDF.amide
[BAT]G.Apc.GDV.Apc.GDFK.amide



60. A reagent for preparing a thrombus imaging agent for imaging a thrombus within a mammalian body comprising a specific binding peptide having an amino acid sequence of 4 to 100 amino acids and a technetium-99m binding moiety covalently linked to the specific binding peptide, wherein the peptide is selected from the group consisting of linear and cyclic peptides that are ligands for a GPIIb/IIIa receptor and do not comprise the amino acid sequence (arginine-glycine-aspartate), peptides that are ligands for a polymerization site of fibrin, and cyclic peptides comprising the amino acid sequence (arginine-glycine-aspartate).

61. The reagent of Claim 60 wherein the amino acid sequence of peptides that are ligands for a polymerization site of fibrin comprise multiple copies of the sequence (glycyl-prolyl-arginyl-prolyl).

62. The reagent of Claim 60 having the formula:

$[(GPRP)_2K]_2KC_{Acm}GC_{Acm}$.amide
$(GPRVVERHQSA)_2KC_{Acm}GC_{Acm}$.amide
$(GPRPC_{Acm}GC_{Acm}C)_3$-TSEA
$[GPRPPPGGC_{Acm}GC_{Acm}GGC]_3$-TSEA
*acetyl*-CNP.Apc.GDC
[BAT].Hly.GDP.Hly.GDF.amide
[BAT]G.Apc.GDV.Apc.GDFK.amide

OC---$Y_D$--RG--D
$H_2C$----S-------C---$C_{Acm}GC_{Acm}$.amide

OC---$Y_D$--RG--D
$H_2C$----S-------C---$WGGC_{Acm}GC_{Acm}$.amide

OC---$Y_D$--RG--D
$H_2C$----S-------C--- $FGGC_{Acm}GC_{Acm}$.amide

OC---$Y_D$--RG--D
$H_2C$----S-------C---$GGGC_{Acm}GC_{Acm}$.amide

OC---$Y_D$--RG--D
$H_2C$----S-------C---$DGGC_{Acm}GC_{Acm}$.amide

OC---$Y_D$--Apc.G-D
$H_2C$----S-------- C---$GGGC_{Acm}GC_{Acm}$.amide

OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide

OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---KGC$_{Acm}$GC$_{Acm}$.amide

OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---GGC$_{Acm}$GC$_{Acm}$GGC.amide

OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---GGC$_{Acm}$GC$_{Acm}$GGC$_{NES}$.amide

{ OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---GGC$_{Acm}$GC$_{Acm}$GGC.amide }$_3$ -TSEA

OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---GGC$_{Acm}$GC$_{Acm}$GGF$_D$PRPG.amide

OC---Y$_D$--RG--D
H$_2$C----S-------C---GGC$_{Acm}$GC$_{Acm}$.amide

OC---Y$_D$--KG--D
H$_2$C----S-------C---GGGC$_{Acm}$GC$_{Acm}$.amide

OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---KGC$_{Acm}$GC$_{Acm}$GGC.amide

Pic.GC$_{Acm}$GG.C.NP.Apc.GDC
S——————S

*acetyl* - C$_{Acm}$GC$_{Acm}$.C.NP.Apc.GDC
S——————S

{ OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---GGC$_{Acm}$GC$_{Acm}$GGC.amide }$_2$ -BSH

{ OC---Y$_D$--Apc.G-D
H$_2$C----S--------C---GGC$_{Acm}$GC$_{Acm}$GGC.amide }$_2$ -BSME

*acetyl*-G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide
G.Apc.GDV.Apc.GDFKC$_{Acm}$GC$_{Acm}$.amide
G.Apc.GDVKC$_{Acm}$GC$_{Acm}$.amide

63. The reagent of Claim 60 that is radiolabled with technetium-99m.

64. The reagent according to Claim 60 wherein the specific-binding peptide and the technetium-99m binding moiety are covalently linked through from about to about 20 amino acids.

65. A complex formed by reacting the reagent according to Claim 60 with technetium-99m in the presence of a reducing agent.

66. The complex of Claim 65, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

67. A complex formed by labeling the reagent according to Claim 60 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

68. A composition of matter comprising the reagent according to Claim 60 and a stannous ion.

69. A kit for preparing a radiopharmaceutical preparation, said kit comprising a sealed vial containing a predetermined quantity of a reagent according to Claim 60 and a sufficient amount of reducing agent to label said reagent with technetium-99m.

70. A method for labeling a reagent according to Claim 60 comprising reacting the reagent with technetium-99m in the presence of a reducing agent.

71. The method of Claim 70, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion and a ferrous ion.

72. A method for imaging a thrombus within a mammalian body comprising administering an effective diagnostic amount of a reagent according to Claim 65 and detecting the technetium-99m localized at the thrombus.

73. The reagent according to Claim 60 wherein the specific-binding peptide is chemically synthesized *in vitro*.

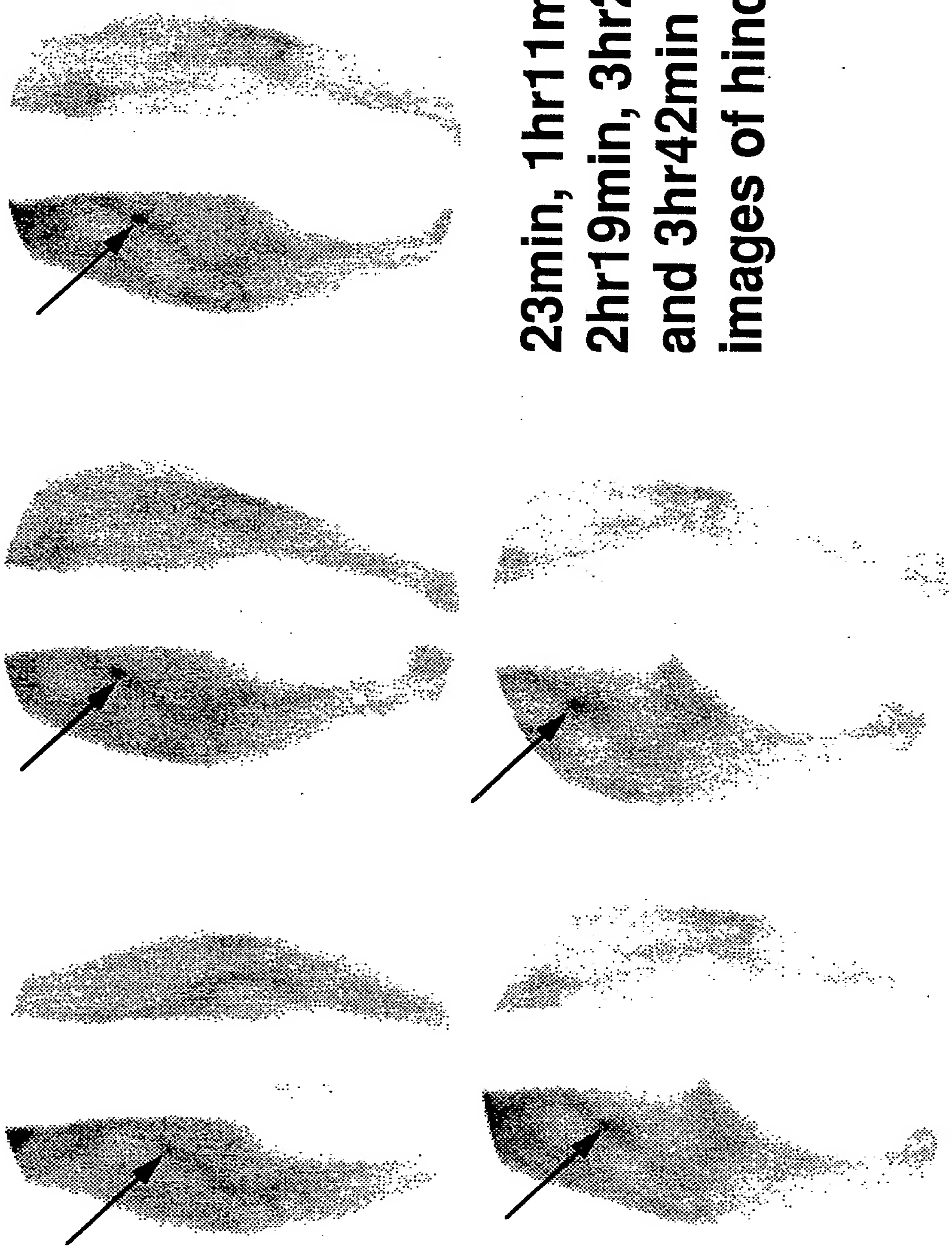
74. The specific-binding peptide according to Claim 73 wherein the peptide is synthesized by solid phase peptide synthesis.

75. The reagent according to Claim 73 wherein the technetium-99m binding moiety is covalently linked to the peptide during *in vitro* chemical synthesis.

76. The reagent according to Claim 75 wherein the technetium-99m binding moiety is covalently linked to the peptide during solid phase peptide synthesis.

77. The reagent of Claim 60 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

78. The reagent of Claim 77 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N*_,*N*_-*bis*(2-succinimido-ethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine, *bis*-succinimidohexane, 4-(O-$CH_2$CO-Gly-Gly-Cys.amide)acetophenone or a derivative thereof.



23min, 1hr11min, 2hr19min, 3hr28min and 3hr42min images of hind legs

Fig. 1

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/04794

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K49/02

**II. FIELDS SEARCHED**

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | A61K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| P,X | WO,A,9 213 572 (DIATECH, INC.) 20 August 1992 cited in the application see the whole document | 1-19,60, 63-76 |
| E | WO,A,9 310 747 (DIATECH, INC.) 10 June 1993 cited in the application see the whole document | 22-37, 40-41 |
| X<br>Y | WO,A,9 010 463 (NEORX CORPORATION) 20 September 1990 see abstract see page 6, line 29 - page 18, line 32 see page 29, line 33 - page 30, line 10 see page 37, line 6 - line 13 see page 40, line 26 - page 41, line 31; claims 1-5,7,13-14,33,57,63; example 1 | 22-32, 34-37<br>33,40-41 |

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 30 SEPTEMBER 1993 | 07. 10. 93 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | HOFF P.J. |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
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| X | WO,A,8 910 759 (MALLINCKRODT) 16 November 1989 see page 2, line 29 - page 9, line 5 see page 13, line 4 - line 26; claim 1 | 42-51 |
| A | EP,A,0 163 119 (THE JOHNS HOPKINS UNIVERSITY) 4 December 1985 see the whole document, in particular page 2 lines 30-32 | 42-51 |
| X | WO,A,9 015 818 (ANTISOMA) 27 December 1990 cited in the application | 22-37 |
| A | see the whole document in particular page 2 lines 5-30 | 1-21, 38-78 |
| A | BIOCONJUGATE CHEMISTRY vol. 1, no. 1, 1990, pages 132 - 137 K.E. BAIDOO ET AL. 'SYNTHESIS OF A DIAMINEDITHIOL BIFUNCTIONAL CHELATING AGENT FOR INCORPORATION OF TECHNETIUM-99M INTO BIOMOLECULES' see the whole document | 43-59 |
| A | INORGANIC CHEMISTRY vol. 29, no. 16, 1990, pages 2948 - 2951 N. BRYSON ET AL. 'PROTECTING GROUPS IN THE PREPARATION OF THIOLATE COMPLEXES OF TECHNETIUM' see the whole document | 43-59 |
| A | WO,A,9 205 154 (MALLINCKRODT) 2 April 1992 see abstract; claims; examples | 43-59 |
| A | WO,A,9 117 173 (CYTOGEN CORPORATION) 14 November 1991 cited in the application see abstract; claims; tables V-VI | 1-78 |

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# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/04794

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|---|---|---|
| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| A | WO,A,8 902 752 (GENENTECH, INC.) 6 April 1989 cited in the application see abstract; claims | 1-78 |
| A | EP,A,0 453 082 (HYBRITECH INC.) 23 October 1991 see abstract see page 4, line 11 - page 5, line 57; claims; example 1 | 20-21, 38-39, 56-57, 77-78 |

Form PCT/ISA/210 (extra sheet) (January 1985)

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9304794
SA 74715

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/09/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
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| WO-A-9213572 | 20-08-92 | AU-A- | 1411792 | 07-09-92 |
| WO-A-9310747 | 10-06-93 | AU-A- | 3276093 | 28-06-93 |
| WO-A-9010463 | 20-09-90 | US-A- | 4986979 | 22-01-91 |
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| | | EP-A- | 0381713 | 16-08-90 |
| EP-A-0163119 | 04-12-85 | US-A- | 4638051 | 20-01-87 |
| | | AU-A- | 4177785 | 07-11-85 |
| | | CA-A- | 1225996 | 25-08-87 |
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